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METHUEN'S
MONOGRAPHS ON
BIOLOGICAL SUBJECTS

General Editor: KENNETH MELLANBY, C.B.E.

HISTOCHEMICAL TECHNIQUE

Histochemical Technique

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Preface

Through its integration of studies on structure and chemical composition, histochemistry can contribute to many biological investigations and provide further insight into the nature of various physiological and pathological processes. The effective use of histochemical methods requires an appreciation of their potentialities and limitations as well as an adequate knowledge of their chemical bases. Mindful of these requirements, this monograph attempts to provide an introduction to the principles and practice of microscopical histochemistry, especially the chemical aspects, and to present a beginning selection of reliable techniques applicable to a variety of biological problems.

I am very grateful to Prof. Charles H. Best, University of Toronto, and Dr. John R. Baker, University of Oxford, in whose laboratories my histochemical investigations have been carried out, supported by research grants and fellowships from the National Research Council of Canada. During the preparation of this book, Dr. S. McGee-Russell provided full details of his then unpublished studies on methods for calcium. The entire manuscript was read by Prof. E. Schönbaum and Dr. K. F. A. Ross who made many helpful suggestions. My associations with Dr. M. Abercrombie, general editor of Methuen's Monographs on Biological Subjects, has been especially pleasant. My wife has been a wonderful support and help throughout the preparation of the book.

W. G. B. C.

Toronto, Canada
April 1958

Before this monograph was reprinted, the Selected Bibliography for each chapter and the list of Additional References at the end of the book were brought up to date.

W. G. B. C.

Palisades, N.Y.
August 1962

CHAPTER 1

Microscopical Histochemistry

Histochemistry is concerned with the chemistry of cells and tissues. It deals not only with the chemical nature of their structural components but also with the nature of the chemical processes going on in them. Sometimes, a distinction is made between 'histochemistry' and 'cytochemistry' on the basis of the level of organization being studied. However, many histochemical observations must be made at a cytological level so that 'histochemistry' properly includes 'cytochemistry' (Baker, 1952).

Histochemistry can be regarded as an extension of morphological investigations to include chemical composition. While histological preparations demonstrate the structural organization of cells and tissues, histochemical preparations enable the identification and localization of certain classes of chemical substances. At the same time, histochemistry resembles biochemistry in its concern with chemical constitution but it differs from biochemistry in being also concerned with morphological localization. The name 'physiological histochemistry' (Dempsey and Wislocki, 1946) emphasizes an aspect of histochemistry that is often overlooked. The greatest value is obtained from histochemical investigations when the observations on structure and composition are correlated with the functional state of the cells and tissues.

The ultimate aim of histochemistry is to describe the dynamic organization of cells and tissues in terms of their structure, composition, and function. The results achieved up to the present fall far short of this goal whose attainment depends upon at least three lines of investigation (Danielli, 1947):

(*a*) determination of the nature, distribution and concentration of the chemical constituents of living cells and tissues.

(*b*) determination of the nature of the chemical reactions

occurring in cells and tissues and the means whereby materials and energy are stored in them, and

(*c*) determination of the mechanisms for the functional utilization of energy provided by the chemical reactions, that is, the fundamental relationships between structure and function in cells and tissues.

1.1 Microscopical histochemistry

A wide variety of physical, chemical and biological techniques are available for studying chemical constituents in their natural locations in cells and tissues. Those methods in which the final observations are made microscopically on tissue sections or similar preparations constitute *microscopical histochemistry*, the subject of this monograph. This branch of histochemistry provides almost the only approach to certain biological problems: because the structural integrity of the cells and tissues is preserved and because the cells or their components can be taken as the units of investigation, information that would be completely lost in preparations such as homogenates can be obtained concerning individual cells or different types of cells in heterogenous populations.

The early development of microscopical histochemistry has been outlined by Baker (1943) and by Pearse (1953). Raspail (1825, 1830) first recognized its possibilities. He devised methods for various organic and inorganic constituents as well as the techniques of frozen sectioning and microincineration. By the middle of the nineteenth century many microanatomists were studying both the structure and the chemical composition of tissues. With the introduction of stains, histochemistry was almost completely abandoned in favour of a parachemistry based on staining properties. Mann (1902), however, published his important *Physiological Histology* during this period. Renewed interest in microscopical histochemistry was heralded by Lison's *Histochimie Animale* in 1936. Since then, histochemistry has developed steadily and rapidly. During the past two decades, innumerable investigations have been published and there are now several textbooks and journals devoted to histochemistry (see Bibliography on page 12).

Microscopical histochemistry is still in a relatively early stage of development. Some of the methods in use and some of the

conclusions reached in studies with them cannot withstand critical analysis (Gomori, 1950). Other methods, even though relatively non-specific, can serve useful purposes provided that the factors influencing the results are known and taken into consideration.

1.11 Limitations. All the methods of microscopical histochemistry have their limitations and it is important that these be recognized. Some of the limitations and sources of error result from the very conditions that characterize microscopical histochemistry. Only static or fixed constituents can be studied when chemical tests are applied to fixed tissues. In cells and tissues, especially in thin sections of them, the amounts and concentrations of some substances are often near, if not below, the limits of detection of the test. Sometimes the size of the particles of the constituents is near or below the limit of microscopical resolution. During the preparation of the tissues and during the performance of the tests, there are dangers of errors being introduced by adsorption, diffusion, solution, denaturation or inactivation with resultant alterations in location, chemical reactivity, or both. Because of technical difficulties such as those associated with fixing, dehydrating, embedding and sectioning the majority of histochemical investigations tend to be descriptive and qualitative. Further difficulties are introduced by the complex chemical nature of cells and tissues; for example, the demonstration of one constituent or one class of constituents must often be attempted in the presence of other substances that might interfere with the test or its interpretation.

1.12 Criteria of accuracy. An additional problem impeding progress in microscopical histochemistry has been the lack of exact criteria of accuracy with which to assess a method and to determine its limitations. A somewhat useful, albeit arbitrary, basis for assessment has been that 'those methods are useful which yield results that are in essence in agreement with, or confirmed by, directly related data obtained by means of simpler, chemically valid or physiological (functional) techniques' (Gersh, 1941). Some of the general characteristics of reliable histochemical methods have been defined (Mann, 1902; Prenant, 1910; Parat, 1927) so that, now, there are established criteria of histochemical validity (Lison, 1936, 1953):

(*a*) Morphologically, there should be precise *localization* of

the substance sought and excellent preservation of the *structural integrity* of the cells and tissues being studied.

(*b*) Chemically, the *mechanism*, the *sensitivity*, and the *selectivity or specificity* of the test should be known.

The general significance of these requirements is discussed in the following chapters on the preparation of tissues for histochemical studies and on histochemical analysis.

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CHAPTER 2

Histochemical Analysis

Most, if not all, of the applications of microscopical histochemistry* in biological research are analytical, being concerned with the detection or determination of substances in their natural locations in cells and tissues. The many problems that can arise in such studies require that the methods employed have sound scientific bases. These problems originate partly in the variety of substances that occur in tissues and partly in the varied conditions under which they are present. As a result, a method that is reliable under one set of circumstances could fail completely under other conditions. Such variations must be taken into consideration when choosing a suitable method.

2.1 Some general considerations of chemical analysis

The success of chemical analyses, whether histochemical or other, depends upon practical experience and precisely specified methods. Moreover, the theoretical basis for each method should be known so that the method can be used intelligently and so that new or improved versions can be developed. Progress in histochemical analysis as in other forms of qualitative analysis requires regular review of all those aspects of chemistry that might lead eventually to developing more sensitive and more reliable procedures.

Chemical analysis ultimately depends upon being able to convert the substance to be determined or detected into another characteristic compound. While there are innumerable reactions resulting in such changes, only certain ones are suitable for analytical applications and the requirements to be met depend upon whether the analysis is to be quantitative or qualitative.

* In this and subsequent chapters, 'histochemistry' is used synonymously with 'microscopical histochemistry'.

A quantitative determination requires that there be practically complete and rapid conversion of the material to be estimated, that the reaction be stoichiometric, and that the amount of either the reaction product or the reagent used be measurable. Most histochemical investigations, however, are qualitative. The requirements for qualitative detection are quite different from those for quantitative determination. Neither completeness of reaction nor measurability is essential for a qualitative test. Instead, the results of the reaction must be observable readily and without doubt. A qualitative method must have adequate sensitivity and selectivity or specificity. The infallibility of the method becomes especially important in analysing mixtures such as tissues.

2.11 Sensitivity. The characterization of chemical tests in terms of their *sensitivity* has been discussed by Feigl (1940, 1943). The sensitivity of a test can best be expressed in terms of its identification and concentration limits. The *identification limit* is the least quantity of material that can be just detected by a given reaction. The *concentration limit* is the lowest concentration in which the material can be present and still be detected by the reaction. Both characteristics of a reaction must be considered in judging its sensitivity. A truly sensitive test must be both quantity- and concentration-sensitive. Unfortunately, so far, very little attention has been paid to these characteristics of histochemical methods even though often an important requirement is high sensitivity. Lison (1953) does mention identification limits occasionally.

2.12 Specificity or selectivity. Regardless of the importance of sensitivity in evaluating a method for qualitative analysis, this should not be the sole basis for its appraisal. Seldom if ever will a histochemical test be applied to a single pure substance. For this reason, the selectivity or specificity of a method is also of great importance. A method demonstrating only one material is said to be *specific* while a method leaving a narrow choice between several substances is said to be *selective.* Accordingly, methods are either specific or not, but the latter can be more or less selective depending upon the number of choices. Most, if not all, histochemical methods are selective rather than specific. Sometimes, it is possible to increase their selectivity by inactivating or removing certain materials that are present and give similar

reactions. This is illustrated by blocking (page 48) and extraction (page 57) methods.

2.13 Limiting Proportions. In qualitative chemical analysis it is often of considerable importance that presumably indifferent substances can affect the sensitivity of a given test, rarely causing an increase but usually some decrease in sensitivity. Quite often, the phenomenon is evident only when the substance sought is present in low concentration. The reason for this reduction in sensitivity is not known. In addition to reducing the sensitivity of a test, indifferent substances can also extend the range of concentrations over which an uncertain result is obtained. Schoorl (1907) first called attention to these effects in connection with microchemical tests. He introduced the term *limiting proportion* to designate the ratio of the quantity of material that could be just detected to the quantity of the attendant indifferent material that could be present without affecting the sensitivity of the test.

In histochemistry, as in analytical chemistry, presumably indifferent substances can affect the sensitivity of a given test and almost invariably decrease it. Unfortunately, the phenomenon has not been studied in any detail and has been too often overlooked. The proteins are frequently assumed to be indifferent substances whereas they can reduce the sensitivity of a test or even react with one of the reagents (see pages 54 and 77).

2.2 Some general considerations of histochemical analysis

2.21 Requirements for a satisfactory method. Histochemical reactions must satisfy not only the requirements of satisfactory analytical reactions but also certain additional requirements imposed by the conditions for a reliable histochemical method (page 11). This section will be limited to a consideration of qualitative histochemical methods. It should be noted, however, that an important principle often overlooked in quantitative histochemical studies is that the requirements for a satisfactory quantitative method in analytical chemistry are quite different from those for a suitable qualitative technique (page 18). Nevertheless, in some histochemical studies quantitative measurements have been attempted using a qualitative method without clearly establishing that it fulfils the criteria of a satisfactory quantitative reaction. Various problems in connection with quantitative histochemical studies are dealt with by Eranko (1955).

Whether in analytical chemistry or in histochemistry, a satisfactory qualitative reaction must have adequate sensitivity and selectivity and favourable limiting proportions for potentially interfering substances. Considering the conditions under which the test is carried out and the necessity of accurate localization, a satisfactory histochemical method must meet additional requirements; namely,

(*a*) The reaction must be applicable to cells and tissue sections; that is, the reaction must be given by the constituents as they occur in the cells and tissues and it must not cause any structural damage.

(*b*) The reaction must be of such a nature and have such a velocity that there is no dislocation of the constituent under investigation and the site of the reaction product coincides with the original site of the constituent.

(*c*) The optical properties of the reaction product must be such that it can be readily studied microscopically. Usually, it is desirable that the product be intensely coloured. Many colour reactions that are satisfactory in analytical chemistry fail to meet this requirement in microscopical histochemistry because of the very much shorter optical path, a few microns in a tissue section instead of several millimetres in a test-tube.

(*d*) It is desirable that the reaction product be stable because the histochemical preparation might need to be studied repeatedly and compared with preparations made at other times. There is a tendency, however, for undue emphasis to be placed on the requirement of permanence of histochemical preparations. If a permanent record is desired, often a coloured photomicrograph will serve admirably.

(*e*) If the product is crystalline, it should be formed under conditions yielding as small crystals as possible so that its localization can be established precisely.

(*f*) Because one of the fundamental objectives of histochemical investigations is to study the constituents of cells and tissues in their normal locations, it is essential that the cells and tissues be handled in such a way that the localization of the constituents does not change during the preparation of the cells and tissues or during the conduct of the test.

Unfortunately, still too seldom are all these special requirements

met by a given histochemical method. Meanwhile, they can serve to guide the development of new techniques. In the past, failure to recognize these requirements has led to the development of completely worthless methods supposedly localizing substances for which no wholly reliable method is yet known.

2.22 Limitations of histochemical identification. There is an important difference in the amount of information that can be provided by chemical and histochemical analysis. The exact substance can often be identified by qualitative inorganic or organic analysis. Identification by histochemical analysis is usually limited to establishing the class of the substance. To break down such generic classes into specific molecular species in analytical chemistry requires techniques unavailable or unsuitable for histochemical analysis. In organic analysis, for example, chemical reactions are used to identify a substance down to a group of closely related compounds. The actual identification of the purified substance then, most often, depends upon one or more of its physical properties rather than upon further chemical reactions. In histochemistry, the substance can never be assumed to be pure and it is seldom possible to determine its physical properties sufficiently precisely in tissue sections.

This limitation of identification by histochemical analysis is reflected in the histochemical classification of substances that can be studied in cells and tissue sections. As will be seen in later chapters, the biochemical systems of classification can rarely be applied directly in histochemical analysis. This is because in biochemical studies, tissue constituents need not be studied in their natural locations but can be isolated and characterized in more detail than is possible histochemically.

2.3 Methods of histochemical analysis

2.31 Classification. Many of the methods of microscopical histochemistry have been taken from other fields of chemical investigation and modified to meet the special requirements of histochemical techniques. An increasing number of methods, however, are being developed especially for histochemical investigations. Most histochemical methods depend upon the chemical reactivity of the substances under study although some depend upon

physical properties. Consequently, the methods can be classified as chemical, quasi-chemical, and physical:

(*a*) The *chemical* methods often employ the same fundamental reactions as are used in analytical chemistry modified, when necessary, to yield an intensely coloured instead of colourless reaction product.

(*b*) The *quasi-chemical* tests are often cytological or histological staining methods that have some histochemical significance. Usually, they are empirical techniques whose mechanisms are not known. Commonly used quasi-chemical staining methods include Best's carmine test for glycogen (page 100) and Mayer's mucicarmine and mucihaematein tests for certain mucins (page 105).

(*c*) The *physical* methods can be subdivided according to the particular property upon which they depend such as solubility, fluorescence or radioactivity. The only physical method considered in detail in this monograph depends upon the solubility of oil-soluble colorants in lipids (page 68).

2.32 Validity. The methods of microscopical histochemistry must be used critically and cautiously in the analysis of constituent tissues and cells. The close resemblance of histochemical methods to histological and cytological staining techniques, the ease with which many of histochemical tests can be performed, the appeal of the often vividly coloured preparations, and the obvious significance of the apparent localizations have all led some investigators unhesitatingly to assume the validity of the methods they have used. However, it is essential that the validity of every technique be established as accurately as possible (Danielli, 1946; Baker & Sanders, 1946). The following questions, modified from those of Danielli (1946) need to be considered carefully:

(*a*) Is the location of the substance under investigation the same in the fixed preparation as in the living cell or tissue?

(*b*) How much of the substance was lost during the preparation of the cells or tissue and how much is destroyed during the test?

(*c*) Do the loss and destruction occur equally at all sites or do they occur selectively?

(*d*) How sensitive and how selective is the method?

(*e*) Does the reaction product remain localized at the sites of the substances under investigation or does the product become affixed to sites having high affinities for it regardless of the location of the substances? At the cytological level even minute displacements can result in significantly false localizations but methods that fail at this level can still be helpful in studying the distribution of materials in tissue and organs.

Establishing the validity of some histochemical methods presents almost no problems, especially methods depending upon highly selective or specific reactions. One of the best examples of such a method is Perls' Prussian blue test for iron (page 158). The difficulties that can arise in attempting to establish the validity of some other methods are well illustrated by the long controversy that has existed regarding the nucleal test for deoxyribonucleic acids (page 113).

Sometimes, in attempting to establish the validity of a particular method, special studies are carried out on pure substances or on models. The use of pure substances in testing histochemical methods presents several problems. Practically it is often difficult, if not impossible, to obtain the pure substances. More serious is the fact that in tissues, substances rarely if ever occur in the pure state, but are almost invariably mixed or chemically combined with other compounds. Moreover, quite frequently the substances in cells and tissues undergo chemical changes during fixing and further processing. Thus, tests on pure substances can be unrealistic and run the risk of giving misleading results.

The purpose of models is to provide conditions more nearly resembling those in tissue sections. One of the first techniques (Altmann, 1890) was to impregnate paper. Pith has also been used in this way (Kaufmann & Lehmann, 1926). Some investigators have dispersed or dissolved the test material in agar or gelatin. The technique introduced by Coujard (1943) is popular at present. He disperses the test substance in dilute gelatin, egg albumin or serum, draws lines with the mixture across a clean slide and then fixes and treats the slide as a tissue section. A number of model experiments have been carried out on histochemical methods for lipids (Altmann, 1890; Mulon, 1904; Camus & Pagniez, 1905; Smith & Mair, 1909; Escher, 1919; Kaufmann & Lehmann, 1926, 1926*a*, 1928; Baker, 1947; Cain,

1948; Casselman, 1953) and carbohydrates (Glegg, Clermont & Leblond, 1952; Staple, 1957). The results of these and similar studies on other methods must be interpreted cautiously. In almost all cases, the 'pure' substances have been biological concentrates whose purity is uncertain at best. Nevertheless, the results do show what reactions can be expected. All such attempts to establish the validity of a method using pure substances or models suffer from the inevitably inductive nature of the proofs. As Cain (1950) has suggested 'there may always be some hitherto unknown compound in some special gland of some undescribed animal which, could we but know it, would give a positive result with the test'.

2.33 Controls. Some tests require control slides in much the same way that certain methods of chemical analysis require blanks. Control slides aid in distinguishing between true, positive reactions and non-specific, falsely positive reactions. In addition to control slides, blocking (page 48) and extraction methods (page 56) are helpful in establishing the significance of positive reactions.

2.4 Histochemical investigations

The histochemical investigation of biological problems requires not only techniques, their application, and the interpretation of the results, but also a particular point of view. It requires thinking of cells and tissues in terms of their chemical constitution as well as structural organization and, at all times, with due consideration of their functional activities. Conscious awareness of morphology is essential lest the histochemical investigations become little more than microchemical studies. Conscious awareness of chemical constitution is at the same time necessary lest the investigations become purely cytological or histological studies. While a thorough knowledge of the structure of the cells or tissues under investigation is an important prerequisite of histochemical studies, it sometimes occurs that such studies also reveal new details of morphology not demonstrated by usual staining methods.

The later chapters of this monograph describe *some*, but by no means all of the methods of microscopical histochemistry that have been found useful for the analytical study of cells and tissues. Additional methods are available and new ones are being

described frequently. Each investigator should develop his own 'repertoire' of techniques upon which he can depend but at the same time he should guard against limiting himself to these methods and should always be alert for other techniques better fulfilling his needs. No given set of methods alone is sufficient, and strict adherence to a few techniques is to be condemned as much in histochemistry as in qualitative chemical analysis.

In so far as practical histochemical analysis is concerned, the advice of Gustav Mann (1902) is as true today as it was over 50 years ago:

'Let each cell organ be examined in a systematic way, not by one but many reagents; do not describe only the effects produced by what some call the best fixative, leaving out the effects produced by "bad" ones; give other investigators the benefit of "failures", and thus gradually help to build up a systematic way of investigating cells.'

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CHAPTER 3

Preparation of Tissues

For most histochemical investigations, the preparation of cells and tissues is essentially the same as for cytological or histological studies except that the various processes should be carried out even more carefully and more exactly. Usually, the tissues must be fixed, dehydrated, embedded and sectioned before the test is applied. Afterwards, the sections must be mounted and examined microscopically.

3.1 Fixation

Because most studies are carried out on fixed tissues, fixation occupies a position of fundamental importance in microscopical histochemistry. Even so, it is also one of the most neglected aspects of histochemical technique. Most procedures have been uncritically adopted from cytology or histology and tacitly assumed to give the 'right result', be that what it may. Only some of the general principles and problems of fixation will be outlined here. They are discussed in more detail in the monograph and book by Baker (1950; 1957) and in the review by Wolman (1955). Practical details of tissue fixation can be found in standard books on histochemical or histological methods, particularly Gray's (1954).

The purpose of fixation is to preserve permanently the cells and tissues in as life-like state as possible. As already specified (page 11), this preservation should include not only the structural organization but also the distribution and reactivity of chemical constituents. Such ideal fixation is unattainable with presently available methods. Fixation involves more than preserving tissues in the above sense. The cells and tissues must also be made resistant to the later steps in processing, such as dehydration and embedding, and at the same time, they must be kept in a state suitable for sectioning.

Prerequisite to understanding the effects of fixation is a knowledge of the chemical nature and submicroscopic organization of protoplasm. These have been discussed by Frey-Wyssling (1953) and Heilbrunn (1956). Fixation brings about both physical and chemical changes in cytoplasm. The physical changes result in alterations in the structural organization, both submicroscopic and microscopic, the latter resulting in appearances characterized as 'good' or 'bad' fixation. Additional physical effects, related to phenomena such as diffusion and adsorption, result in changes in localization. Some of the chemical effects, such as denaturation, result in significant physical changes while others lead to significant changes in the chemical properties of the constituents of the tissues. For example, during fixation of a tissue in a mixture containing chromic acid, glycogen can be oxidized to products reacting positively with Schiff's reagent (*cf.* page 32), lipids to products insoluble in the usual fat solvents (Ciaccio, 1912) and deoxyribonucleic acids can undergo hydrolysis initially similar to that in the nucleal test (page 113). Chromic acid can also have various effects upon the morphology of the nucleus and cytoplasm of cells depending to some extent upon the hydrogen-ion concentration of the fixative (Casselman, 1954).

The choice of fixative is important for histochemical studies. It should not only preserve histological and cytological details well, introducing a minimum of artifacts, but it should also not alter the localization or chemical reactivity of the substances to be investigated. Unfortunately, these requirements are difficult to meet because fixation depends to some extent upon producing certain physical or chemical changes or both in at least some constituents of the cells and tissues, notably the proteins, and because reagents that preserve morphology well do not necessarily also satisfactorily preserve the reactivity of the constituents, especially the enzymes. Consequently, it is often necessary to use several fixatives with adjacent blocks of tissue.

Pollister (1952) has emphasized that some fixatives that preserve cell morphology well, such as neutral osmium tetroxide, are of little value for most histochemical tests because in addition to impairing stainability, the reagents inhibit enzymes and render various constituents of the cells resistant to digestion or extraction procedures. On the other hand, fixatives, such as organic solvents, which might not appreciably interfere with chemical

reactivity, do greatly disrupt intracellular structures and can produce significant changes in chemical composition, almost always extracting lipids and disorganizing mitochondria (Palade, 1951). Following fixation by organic solvents, not only are most lipids and water-soluble materials lost, but there is almost certainly always some redistribution of the substances which remain in the cells and tissues, possibly leading to false localization.

In subsequent chapters, the fixatives that have been found to give most satisfactory results will be mentioned in connection with the various methods. Practically, the usual requirements for satisfactory fixation apply; namely, ample fixative, and prompt fixation, although, in some cases, tissues can be held in the refrigerator for a short time without any appreciable changes.

3.2 Embedding

Generally, fixed tissues can be embedded in paraffin or celloidin as in histological technique. Celloidin embedding has the advantage of not requiring heating. This can be important in studies on heat sensitive materials. However, the dilute alcohol in which celloidin blocks are stored tends to inactivate enzymes so that the blocks should be cut and processed as promptly as possible. An abridged procedure such as the following recommended by Gomori (1952) gives good results:

1. Cut thin slices of tissue.
2. Dehydrate in several changes of absolute ethanol or acetone, 1–2 hours each.
3. Transfer to a mixture of equal parts of alcohol and ether for 2 hours.
4. Transfer to 4% celloidin in equal parts of ethanol and ether for 12 or 24 hours.
5. Harden in 70% ethanol for a few hours and transfer to water.

The entire procedure should be carried out in the refrigerator. Tissue blocks infiltrated by this method can be cut readily on a freezing microtome and have no tendency to disintegrate.

Paraffin embedding appreciably inactivates enzymes (Table 10.1, page 164) although completely anhydrous proteins often resist denaturation by heat. The inactivation is accelerated by any traces of water. Therefore, thorough dehydration of the tissue

is essential. Excessive heat should be avoided during paraffin embedding. The temperature should not exceed 56° or 58° C. The time should be as short as possible and can be shortened by vacuum embedding.

Frozen sections such as are used for lipid studies can be cut from many tissues without prior embedding. In some cases, especially with small pieces or fragile tissues, embedding in gelatin is necessary. Alternatively, especially if sections are to be cut on a paraffin-type microtome, the tissues can be embedded in polyethylene glycols (Carbowaxes) (Blank, 1949; Blank & McCarthy, 1950; Firminger, 1950; Rinehart & Abu'l-Haj, 1951; Hale, 1952; Wade, 1952).

3.3 Freeze-drying

The technique of freeze-drying was introduced by Altmann in 1890 but was seldom used during the next 40 years. Gersh (1932) renewed interest in the method. Its particular advantages are that all the solid constituents of tissues are precipitated without extraction or diffusion and that these substances are retained in a reactive state, chemically unchanged (Gersh, 1932). The tissues are frozen virtually instantaneously and then dried without exposure to solvents or chemical reagents. Theoretical aspects of freeze-drying are discussed by Malmstrom (1951). Bell (1952) has reviewed some of the methods and compared the results with those obtained by usual fixation. Good results are being obtained with the apparatus designed by Stowell (1951) and an automatic temperature regulator such as that of Andrew and Hale (1954). More recent designs of freeze-drying equipment are described by Burstone (1956), Freed (1956), and Glick and Bloom (1956).

3.4 Fresh-frozen sectioning

In some instances, fresh tissues must be used because certain constituents are altered or destroyed by fixing and embedding. With ordinary equipment, good sections are difficult to cut and tend to be rather thick for cytological studies. Special methods have been introduced using a modified freezing microtome (Adamstone & Taylor, 1948; Fernandez-Moran, 1952) or a cryostat (Linderstrøm-Lang & Morgensen, 1938; Coons, Leduc & Kaplan, 1951).

Thornburg and Mengers (1957) have recently undertaken to

analyse the factors influencing frozen-sectioning. They have established the optimum temperature relations and have proposed a mechanism of frozen-sectioning involving a zone of 'micro-melting' at the edge of the knife.

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CHAPTER 4

Selective Methods

Certain types of selective methods are used rather widely in histochemical analysis, including aldehyde reagents, selective oxidizing agents, blockade and extraction techniques, selective staining, and esterification. The principles of some of these methods will be outlined in this chapter. Applications of these methods in the study of various constituents of cells and tissues will be discussed in succeeding chapters.

4.1 Detection of aldehydes

4.11 Histochemical importance of aldehydes. Some of the most commonly used histochemical tests depend upon the formation and subsequent detection of aldehydes. The class of compounds demonstrated is determined by the technique used to produce the aldehydes, usually selective oxidation or selective hydrolysis. The substances demonstrated by such tests include certain carbohydrates (PA/S test, page 40), nucleic acids (nucleal test, page 113) and compound lipids (plasmal test, page 81).

In histochemical tests, two types of aldehydes have to be considered: free aldehydes, naturally present in the tissues or introduced during fixation, and aldehydes intentionally formed during the test. Naturally occurring free aldehydes are usually fatty aldehydes, that is, aldehydes related to the fatty acids (page 65). They are incompletely or not at all preserved in fixed tissues especially after dehydration and embedding in paraffin. Such aldehydes are best sought in fresh tissues sectioned on the freezing microtome. Formaldehyde, used for fixation, is mostly removed during the preparation of paraffin sections but must be thoroughly washed out or, better, blocked by a suitable reagent (page 48) when studies are to be made on frozen sections of fixed tissues. Free aldehydes can also be formed from unsaturated lipids during

storage of tissues. Verne (1929) has shown that the oxidation products of unsaturated fatty acids can give positive reactions with Schiff's reagent. The process here is probably the formation of peroxides which then break down to form aldehydes. This is probably the explanation of the positive reaction for oleic acid reported by Lison (1932). In his studies on lipid carbonyl compounds, Gomori (1952, 1952*a*) also demonstrated that abundant aldehydic compounds can be formed by the oxidation of unsaturated lipids in tissues.

None of the carbohydrates in tissues give direct aldehyde reactions. Instead, reactive aldehyde groups must be formed by oxidation or hydrolysis. Polysaccharides, for example, are oxidized by a selective reagent such as periodic acid which converts vicinal hydroxyl groups to aldehydes (see page 40). Although the nucleic acids contain carbohydrate moieties, aldehydes cannot be obtained from them by oxidative methods. In ribonucleic acid, the phosphoric ester linkage on position 3 of the ribofuranose (page 112) blocks oxidation. Any attempt to remove it results in depolymerization of the ribonucleic acid and its loss from the section. In deoxyribonucleic acid, there are no vicinal hydroxyl groups. Controlled hydrolysis with dilute hydrochloric acid as in the nucleal test (page 113), however, removes the purine bases exposing the deoxyribose. This sugar, in contrast to the common pentoses and hexoses, exists largely in a non-cyclic form with a terminal aldehyde group.

Aldehydes are formed from acetalphosphatides by treating these lipids with mercuric chloride or even dilute acid, the plasmal test (page 81).

Controls are important in tests involving aldehydes, especially in studies on unfamiliar material, because of the many, potential sources of aldehydes including the fixative if it has not been completely removed, aldehydes naturally present in the tissues, and aldehydes formed either intentionally or incidentally during the test.

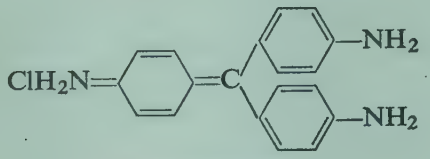
4.12 Schiff's reagent. Theoretically, any of the numerous aldehyde reactions used in organic chemical analysis could be used for the histochemical detection of aldehydes. In practice, only a few reagents meet the requirements of histochemical tests. The most important of these is Schiff's (1866) reagent.

It is generally agreed that the groups reacting with Schiff's reagent in tests for deoxyribonucleic acids, polysaccharides and acetalphosphatides are aldehydes. Schiff's reagent is not specific for aldehydes, however, and also forms coloured products with some ketones. Feulgen, Imhaeuser and Behrens (1929) named the type of reaction with Schiff's reagent given by acetone, a 'pseudo reaction' assuming that the reaction product was different from that obtained with aldehydes because the colour is reddish rather than purple. Oster and Mulinos (1944; Oster & Oster, 1946) suggested that the 'true' positive reaction for aldehydes could be differentiated from the 'pseudo' positive reactions of other carbonyl compounds by treatment with alkali. Both are decolorized by sodium hydroxide. Only the aldehyde reaction is restored by subsequent treatment with hydrochloric acid.

'Recolorization' of Schiff's reagent is a term that should be used cautiously and not as a synonym for the production of an aldehyde addition compound. Recolorization should be limited to those instances where the Schiff's reagent is restored to the original basic fuchsin. This occurs under conditions resulting in the removal or oxidation of the sulphurous acid or in any appreciable decrease in the strong acidity of the reagent. Except with acid-fast structures, the basic fuchsin formed by recolorization can be extracted by acids or alcohol whereas the aldehyde complex resists both reagents.

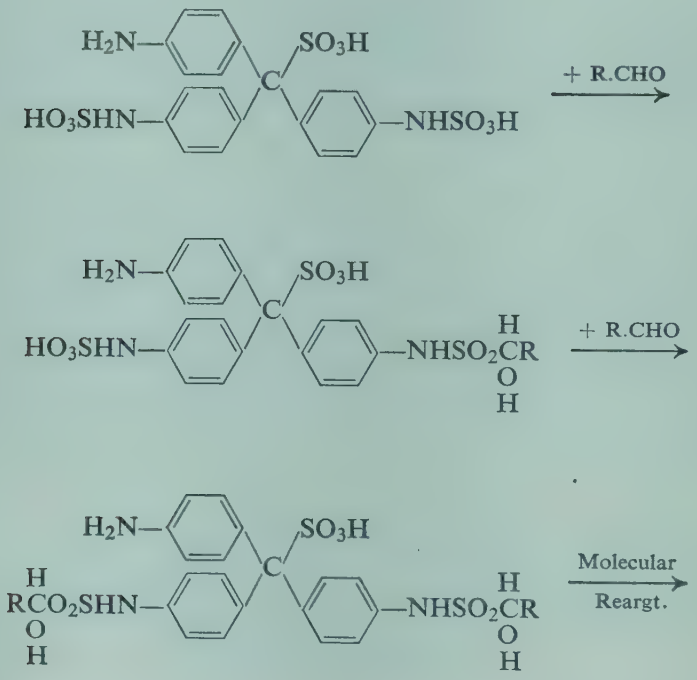
4.121 Chemistry. When basic fuchsin is treated with sulphurous acid, a colourless product is obtained, known as Schiff's reagent. It is also called leuco-basic fuchsin or fuchsin-sulphurous acid, but because the reagent is not the simple reduction product of basic fuchsin, 'leuco-basic fuchsin' is an inaccurate and undesirable name. 'Fuchsin-sulphurous acid', however, is acceptable.

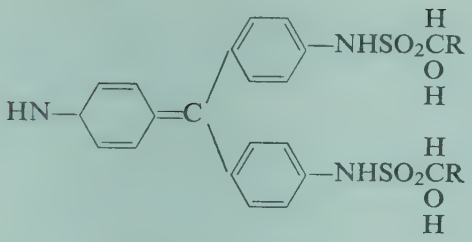
Commercial basic fuchsin (C.I.677) is a mixture of pararosaniline and its mono- and di-methyl derivatives, rosaniline and



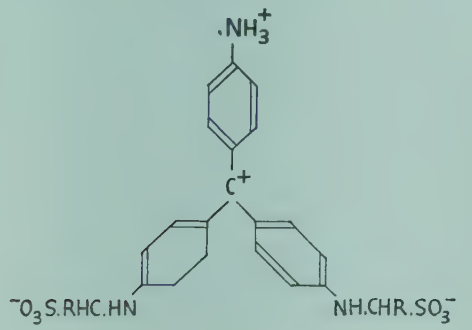
(Pararosaniline)

magenta II respectively (Conn, 1953). The parent dye, pararosaniline or magenta O, is the principal constituent of basic fuchsin. It is generally present as the acetate, sometimes as the chloride. Because of its quinoid structure, the dye is relatively unstable, being readily oxidized with consequent destruction of the molecule. The dye can be reduced to the leucobase or converted by alkali to the colourless carbinol. With sulphurous acid, however, this simple reduction does not occur. Instead, during the preparation of Schiff's reagent (Wieland & Scheuing, 1921) the pararosaniline is first converted to the colourless leucosulphonic acid by the 1,6-addition of sulphurous acid to the quinoid nucleus of the dye. Additional sulphur dioxide then reacts with the leucosulphonic acid yielding the bis-N-aminosulphinic acid that is Schiff's reagent. It reacts with two molecules of aldehyde to produce a reddish-purple addition complex. The exact mechanism is uncertain but Wieland and Scheuing (1921) have suggested that following the addition of two molecules of aldehyde, the complex





undergoes molecular rearrangement to form an intensely coloured quinoid complex. While the studies of Wieland and Scheuing are almost exclusively cited in the histochemical literature, the investigations of Rumpf (1935) should not be overlooked. He has suggested that the following structure is more in keeping with the known physicochemical properties of Schiff's reagent and its reaction with aldehydes:



In either case, the coloured reaction product is not basic fuchsin. Indeed, its absorption spectrum (Stowell & Alberts, 1943) differs from that of the original dye and depends, to some extent, upon the aldehyde. In other words, Schiff's reagent is not 'recoloured' by aldehydes—a new compound is formed.

4.122 Preparation. The procedures recommended by different authors for preparing Schiff's reagent result in products varying widely in dye, hydrogen-ion and sulphur dioxide contents. The effects of these variables have been studied by several investigators

(Ely & Ross, 1949; Atkinson, 1952; Longley, 1952). Atkinson (1952) and Longley (1952) have shown the importance of the concentrations of the dye, sulphite ions and hydrogen ions in the reagent; the method of decolorization as well as the conditions, such as time and temperature, under which the reagent is used. While the optima are often quite sharply defined in spectrophotometric studies, such as these two authors made, they are usually much broader in histochemical tests on tissue sections.

When the initial concentration of dye is low (under 0·125%), not all positively reacting sites will be demonstrated. When it is high (1% and over), the reagent gradually precipitates while stored in the refrigerator. This tendency to precipitate appears to be greater if the dye is dissolved in hot acid. The molar ratio of sulphite to basic fuchsin should be between 5 : 1 and 10 : 1. The pH of the reagent should not be much above 2 as fuchsin-sulphurous acid begins to precipitate at or slightly above pH 3. Atkinson (1952) suggested that the particular reducing agent used in preparing Schiff's reagent appreciably affects the results. Re-investigation of the reducing agents (Casselman, unpublished) all at the same pH, and taking into consideration the amount of SO_2 produced, has shown that they do not differ significantly. Removal of the yellowish impurities by decolorizing charcoal is more effective when carried out after the basic fuchsin has been reduced. The charcoal does remove a small amount of fuchsin-sulphurous acid; therefore, a large excess should be avoided. In most cases, 0·3–0·4% fresh, decolorizing charcoal is effective.

Despite the variations in the procedures recommended for preparing Schiff's reagent, most of them give satisfactory results in histochemical methods. Variations in results are more apt to be due to differences in batches of basic fuchsin or to the age of the prepared reagent rather than to the differences in methods of preparation. A simple procedure giving consistently good results is that of Lillie (1951), as modified by Longley (1952).

1. Dissolve 0·5 gm. basic fuchsin and 0·5 gm. potassium or sodium metabisulphite in 100 ml. 0·15N. hydrochloric acid.
2. Shake the mixture at intervals for 2–3 hours, until the dye has been converted to fuchsin-sulphurous acid.
3. Add 300 mgm. fresh decolorizing charcoal and shake for at least 5 minutes.

4. Filter through fluted, hardened filter paper. The filtrate should be clear and colourless, like water. If not, treat again with charcoal and filter.

Following the conversion of the basic fuchsin to Schiff's reagent by treatment with metabisulphite and acid, the mixture should be straw yellow or light brown, possibly with some precipitate, depending upon the quality of the basic fuchsin. Treatment with decolorizing charcoal removes the coloured impurities.

In histochemical techniques, it must be remembered that Schiff's reagent is being used as a sensitive analytical reagent. It should be carefully prepared and precisely used. It should not be treated, as is too often the case, like some insignificant staining solution. The reagent tends to be unstable, losing sulphur dioxide upon prolonged exposure to air. With such loss of sulphur dioxide, it gradually reverts to basic fuchsin. This process is hastened by alkali, oxidizing agents or even dilution with water. To prevent loss of sulphur dioxide, the reagent should be kept in a tightly stoppered or screw-capped reagent bottle, preferably well filled. It is advisable to keep the reagent in the refrigerator, even though some of it might precipitate in the cold. To avoid contamination, used Schiff's reagent should not be returned to the stock bottle.

Schiff's reagent, stored in the refrigerator, should be allowed to come to room temperature before use. While more precise regulation of temperature might be theoretically desirable, it is unnecessary in practice. Heating the reagent should be avoided—the equilibrium between dye and sulphite is so altered that a deep violet colour develops. The change is reversible, the colour disappearing on cooling.

4.123 Use. For the results of tests carried out at different times to be comparable, techniques using Schiff's reagent should be carefully standardized. With most aldehydes, maximum colour intensity is reached well within 10 minutes. Prolonged exposure to Schiff's reagent should be avoided when sections have been pretreated with some 'blocking' agents, especially sulphite, because the acidic Schiff's reagent might 'unblock' them (page 48). Moreover, prolonged exposure to the acidic Schiff's reagent can hydrolyse deoxyribose nucleic acid as in the nucleal test (page 113).

The following routine gives consistent results following whatever pre-treatment and washing is required by the test.

1. Immerse section in Schiff's reagent for 10 minutes. There may be very little colour developed following oxidation with chromic acid.
2. Wash off excess reagent with 'sulphite rinse', freshly prepared by mixing 50 ml. 1% potassium or sodium metabisulphite and 50 ml. 0·1N. hydrochloric acid. Do not use water to remove the Schiff's reagent. Recoloration would result (page 34) obscuring sites of positive Schiff reactions.
3. Immerse for 2 minutes in each of 3 successive changes of the above 'sulphite rinse'.
4. Wash in tap water for 5 minutes. This intensifies the Schiff reaction, especially for glycogen and mucin following periodic acid.
5. If desired, stain nuclei with haematoxylin. Nuclear staining is poor after chromic acid. (DO NOT stain nuclei with haematoxylin in nucleal test!)
6. Counterstain cytoplasm if desired.
7. Dehydrate and mount in balsam or synthetic resin.

Many authors have speculated about the significance of differences in the colour developed with Schiff's reagent. Lison (1953) has emphasized that any special interpretations should be made with great caution. Pearse (1953) recommends that any colour be regarded as a positive reaction and that 'departures from the usual magenta should be regarded as having unknown but possibly interesting significance'. Perhaps differences in hue can be related to differences in the aldehydes. Gomori (1950) has clearly demonstrated this for aromatic aldehydes and the same is true for aliphatic aldehydes (unpublished observations). Hydrogen-ion concentration is the only other factor known with certainty to influence the colour.

4.2 Selective oxidants

Despite its unpretentious introduction for the 'histological demonstration of mucin' (McManus, 1946), the periodic acid/Schiff test has become an important histochemical procedure. It has led to the use of other oxidizing agents and to the development of certain differential procedures that have greatly extended the scope of tests employing selective oxidants. In most instances, the tests depend upon the formation of aldehydes which are then

demonstrated by Schiff's reagent (page 32). Many of the tests were devised for carbohydrates, the best known being the periodic acid/Schiff test (PA/S test) which was first described by McManus in 1946. Marchese (1947) quickly noted the similarity between it and Bauer's (1933) test and reported that not only mucin but also other polysaccharides give positive PA/S-reactions. The histochemical significance of this test was later acknowledged by McManus (1948). Other methods employing acidified periodate or periodic acid were devised independently by Lillie (1947), for staining reticulum, and by Hotchkiss (1948), for polysaccharides. The latter's unpublished studies actually antedated McManus's first paper. The PA/S test has been further investigated by Lillie (1950) who also compared it with the Casella (1942) and Bauer (1933) techniques (Lillie, 1951).

The use of lead tetra-acetate in place of periodic acid was described in 1951 by Crippa and soon after by Shimizu and Kumamoto (1952), Lhotka (1952), Jordan and McManus (1952), Glegg, Clermont and Leblond (1952), and Hashim and Acra (1953), all working independently. Glegg and his associates used this reagent in their careful analysis of the PA/S test. Meanwhile, Pearse (1951) and Lillie (1952) investigated the histochemical applications of performic and peracetic acids followed by Schiff's reagent. More recently, Lhotka has introduced sodium bismuthate (Lhotka, 1952*a*), certain manganese acetates (Lhotka, 1953) and aryl iodosoacetates (Lhotka, 1954) as selective oxidizing agents.

During the development of methods employing selective oxidants, various alternatives for Schiff's reagent have been suggested. Their value has proven to be largely academic and none has received widespread acceptance for routine studies. A number of differential techniques—enzymic hydrolyses (page 58) and chemical 'blockades' (page 48)—have also been suggested.

The selective oxidants can be divided into two types: those cleaving α-glycol and related groups, for example, periodic acid, lead tetra-acetate, and sodium bismuthate; and those oxidizing ethylenic groups, the organic peracids. An important property of all these selective oxidants is that ordinarily, they do not further oxidize the aldehydes formed initially. In this regard, they differ from other oxidizing agents such as chromic acid and potassium permanganate.

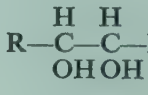
4.21 Periodic acid. The acid usually referred to as 'periodic acid' is, strictly speaking, paraperiodic acid, H_5IO_6. Two salts are commonly available: sodium metaperiodate, $NaIO_4$, soluble in neutral or weakly acidic solutions, and potassium metaperiodate, soluble in neutral or weakly alkaline solutions. Several reviews have been published on applications of periodic acid in organic chemistry (Jackson, 1944) and in biochemistry, with regard to its action on natural products (Courtois, 1948, 1949), and its use in analysis (Dyer, 1956). The selectivities of periodic acid and lead tetra-acetate have been compared by Fleury and Courtois (1950).

The selective action of periodic acid, especially its oxidative cleavage of α-glycols, was first observed in 1928 by Malaprade (1928, 1928*a*). Periodic acid can cleave the carbon–carbon bonds of not only α-glycols, but also α-hydroxyaldehydes, α-ketols, α-ketonealdehydes, and α-diketones, as well as the corresponding amino derivatives.

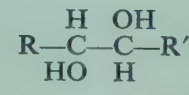
In α-glycols, a primary alcohol group yields formaldehyde while a secondary alcohol group yields formic acid:

$$\begin{array}{l} CH_2OH \\ | \\ (CHOH)_n \\ | \\ CH_2OH \end{array} + (n+1)\,H_5IO_6 \longrightarrow \begin{array}{l} CH_2O \\ + \\ nHCOOH \\ + \\ CH_2O \end{array} + (n+1)\,HIO_3 + (2n+3)\,H_2O$$

Generally, *cis*-α-glycols (I) are oxidized faster than *trans*-α-glycols (II). Similarly, the greater the proportion of *cis*-α-glycol groups they contain, the more rapidly do polyhydroxy compounds tend



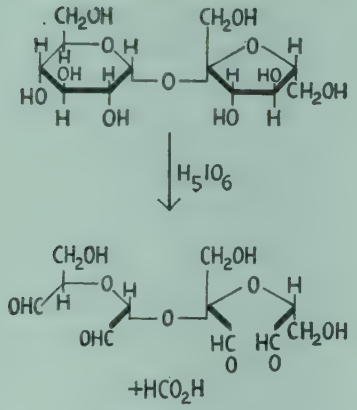
I. *cis*



II. *trans*

to be oxidized. The rate of oxidation is also influenced by other groups in the vicinity of the α-glycol. Large groups tend to retard the oxidation whereas additional hydroxyl groups can accelerate the process not only for α-glycols but also for α-hydroxyaldehydes. Ketoses, on the other hand, are seldom oxidized completely.

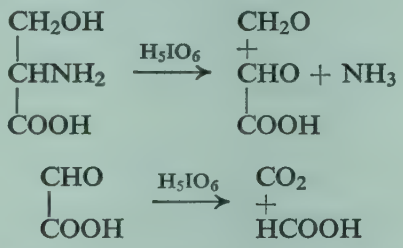
Oxidation by periodic acid does not disrupt the ring structure of aldose acetals and glycosides, as is shown by the following equation for the oxidation of sucrose:



(Sucrose oxidation)

Similarly, ketose acetals and glycosides are oxidized without cleavage of the acetal bond. Although the α-hydroxyacids are not cleaved by periodic acid, the α-ketoacids are oxidized at various rates, determined by the conditions.

α-amino alcohols can be oxidized by periodic acid, *cis*-isomers more readily than *trans*-isomers. Of histochemical interest, is the possible oxidation of serine:



and threonine which can be blocked by N-acylation. Simple α-amino carboxylic acids, however, cannot be oxidized at all. Secondary amines can be oxidized while tertiary amines vary widely in their ease of oxidation.

The possibility of anomalous oxidation by periodic acid needs to be considered more seriously in histochemistry. The influence of temperature, pH, alkali, and concentration of periodic acid will be discussed below. Anomalous oxidations can occur under ordinary conditions. Such oxidations have been described by Dyer (1956) as taking place when 'a substance is oxidized which,

based on structural considerations, should not be, or a substance is not oxidized which, based on structural considerations, should be'. A number of examples of these anomalies are given by Dyer (1956). They fall into three groups: the non-oxidation of α-glycols, the oxidation of active methylene groups, and overoxidation.

The ease of oxidation of α-glycols is influenced not only by the steric relation of their hydroxyl groups but also by the nature of the other parts of the molecule and their spatial relations to the α-glycol groups. There are a number of examples of carbohydrates and their derivatives that fail to be oxidized appreciably by periodic acid under biochemical or histochemical conditions (Jeanloz, 1950). Consequently, failure of periodic acid oxidation even under favourable conditions cannot be taken as unequivocal proof of the absence of α-glycol groups.

Oxidation of active methylene or methine groups is probably of negligible importance in histochemistry. It takes place very slowly under the usual conditions of the PA/S test.

Histochemical conditions are somewhat favourable for overoxidation since there is very great excess of periodic acid in an acid (pH 2) medium. However, there is little risk of overoxidation in the usual PA/S test unless the time in periodic acid is greatly increased. Overoxidation of normally oxidized polysaccharides results in the progressive loss of the terminal residues. 1,3-linked polysaccharides undergo overoxidation more slowly than 1,4-linked polysaccharides while those with 1,6-linkages are relatively stable.

The conditions under which periodic acid is used in histochemistry have not been given much careful attention, yet the extent to which oxidation takes place, the nature and quantity of oxidation products formed, and the occurrence of side reactions as well as overoxidation are all influenced by the conditions.

For glycol cleavage, the concentration of periodic acid should be between 0·01M and 0·1M. For the PA/S test, it is most often 0·02–0·04M. (0·5–1·0%). At concentrations much above 0·1M., the rates of side reactions increase and there is a greater risk of non-specific oxidation. At very low concentrations, the rate of oxidation is unduly slow.

Under histochemical conditions, there is always a very large molar excess of periodic acid. This is but the extreme case of the usual conditions in organic chemistry and biochemistry where

periodic acid is usually present somewhat in excess of the amount required, except when oxidation is to be limited to a particular primary reaction site.

Hydrogen-ion concentration is an important factor influencing oxidation by periodic acid. Aqueous solutions of the acid as commonly used in histochemistry are about pH 2. In biochemistry, a slightly less acid (pH 3–5) medium is considered best for the cleavage of α-glycols, α-hydroxyaldehydes, α-ketols, and α-diketones when there is no stepwise oxidation. With polyhydroxy compounds, such as glucose, where stepwise oxidation can occur, a strongly acid medium (*e.g.* 3N sulphuric acid) favours more rapid oxidation.

The oxidation of α-amino alcohols and the other amino compounds occurs slowly at pH 2 and most rapidly around pH 7–8. However, it is inadvisable to carry out oxidations above pH 5 because side reactions increase greatly. In addition, and of considerable importance in the PA/S test, the aldehydic products of α-glycol cleavage are unstable in alkaline media.

The use of buffered solutions of periodic acid is common in biochemistry. Their use has been rare in histochemistry although worthy of consideration for critical studies. Owing to possible complex formation, phosphate buffers would be better avoided.

Not all reactive groups are oxidized at the same rate by periodic acid. The difference between *cis*- and *trans*-configurations has been mentioned already, and has been made use of by Lhotka (1952*b*). In general, α-glycols and α-amino alcohols are oxidized most readily. The other hydroxy-, keto- and amino-derivatives require longer.

The PA/S test is usually carried out at room temperature. The reaction is accelerated by increasing the temperature but at the risk of overoxidation and increased side reactions. Conversely, at lower temperatures, oxidation proceeds more slowly. Advantage has been taken of this in biochemical studies on aminosugars and polysaccharides. It might be used to advantage in histochemistry for similar studies as well as in distinguishing between *cis*- and *trans*-α-glycol groups.

Oxidation by periodic acid is usually carried out in an aqueous medium. Organic solvents can be used provided they are not appreciably oxidized by periodic acid. Ethanol is used in the general purpose PA/S method introduced by Hotchkiss (1948)

as well as in the special method for dextran of Mowry, Longley and Millican (1952).

The following simple PA/S test illustrates the use of periodic acid as a selective oxidant:

1. Bring sections to water and oxidize in 0·5% aqueous periodic acid for 10 minutes at room temperature. Many tissue constituents containing α-glycol groups give positive reactions after 2–5 minutes' oxidation. Additional structures give positive PA/S reactions after 18–25 minutes' oxidation but such delayed reactions are probably due to groups other than α-glycols.
2. Wash for 5 minutes in running water and rinse in distilled water.
3. Immerse for 10 minutes in Schiff's reagent (page 36).
4. Wash off the excess Schiff's reagent with a freshly prepared mixture of equal parts of 1% potassium or sodium metabisulphite and 0·1N hydrochloric acid.
5. Immerse for 2 minutes in each of three successive changes of the above 'sulphite rinse'.
6. Wash in tap water for 5 minutes.
7. Stain nuclei with haematoxylin, such as Mayer's, and counterstain cytoplasm, if desired.
8. Dehydrate and mount in synthetic resin or balsam.

A positive PA/S reaction is indicated by the red or reddish purple colour characteristics of a positive Schiff reaction (page 38).

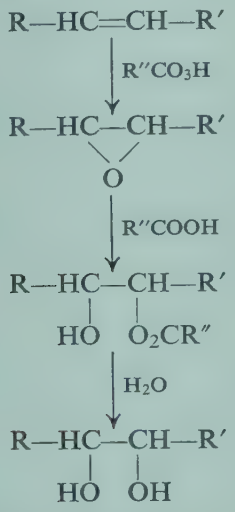
4.22 Lead tetra-acetate. Dimroth, Friedman and Kammerer (1920) were amongst the first to use lead tetra-acetate as an oxidizing agent in preparative organic chemistry. In this field, it has found many applications such as dehydrogenation, replacement of hydrogen by an acetylated hydroxyl group and the addition of two such groups to a carbon–carbon double bond, but, perhaps most important of all is oxidative glycol cleavage which is also the basis of its use in histochemistry. Criegee (1931) described the splitting of the bond between two adjacent, hydroxyl-bearing carbon atoms by lead tetra-acetate to yield aldehydes or ketones. The reaction proceeds under the mildest conditions in dilute acetic acid or in inert solvents. Baer, Grosheintz and Fischer (1939; Grosheintz, 1939) introduced the use of aqueous media for oxidation by lead tetra-acetate.

At first, lead tetra-acetate and periodic acid were considered to be similar in regard to their oxidative potentialities and mechanisms of action. Later it was found that while both react with α-glycols and certain other groups (page 40), only lead tetra-acetate reacts with α-hydroxy acids and that periodic acid will not effect substitution, addition or dehydrogenation reactions. The scope of selective oxidations by lead tetra-acetate has been reviewed by Criegee (1948).

At least seven histochemical techniques have been described in which lead tetra-acetate is used as a selective oxidant and followed by Schiff's reagent as in the PA/S test (Crippa, 1951; Glegg, Clermont & Leblond, 1952; Jordan & McManus, 1952; Shimizu & Kumamoto, 1952; Hashim & Acra, 1953; Graumann, 1953). Not all of these methods give the same results with at least some polysaccharides. Comparative studies (Casselman, 1954) have shown that the differences between the results are related to the conditions under which the lead tetra-acetate is used. Starch and glycogen do not give positive lead tetra-acetate/Schiff reactions when the oxidizing agent is used in glacial acetic acid or in acetic acid diluted with a non-aqueous solvent as in the methods of Crippa (1951), Glegg, Clermont and Leblond (1952), Graumann (1953) and Hashim and Acra (1953). With potassium acetate added as a catalyst in a non-aqueous mixture (Lhotka, 1952), the two polysaccharides give weakly positive reactions, especially following prolonged oxidation. Starch and glycogen give positive reactions following oxidation by lead tetra-acetate in an aqueous medium (Hashim & Acra, 1952) especially with added sodium acetate (Jordan & McManus, 1952; Shimizu & Kumamoto, 1952). Thus, while acetate can act simply as a catalyst, the addition of water not only accelerates the reaction but decreases the selectivity of lead tetra-acetate oxidation.

4.23 Organic per-acids. Performic and peracetic acids have been used to a limited extent as selective oxidizing agents in histochemical studies on lipids (Lillie, 1952, 1954; Casselman, Macrae & Simmons, 1954) and on hair (Pearse, 1951, 1953; Lillie & Bangle, 1954; Lillie, Bangle & Fisher, 1953; Findlay, 1955). The apparent behaviour of these reagents in histochemical tests is not entirely in agreement with their known chemical properties (Swern, 1949, 1953). Thus, when an unsaturated compound is

oxidized by an aliphatic per-acid, an α-glycol is formed through α-epoxy and hydroxy ester intermediates:



(Oxidation by organic per-acid)

The oxidation is supposed to stop at the α-glycol stage, but, under histochemical conditions, products are formed that react with Schiff's reagent. Moreover, their formation is prevented by prior bromination which is presumed to block ethylenic groups (page 51). Although the mechanism of the performic (PFA/S) and peracetic (PAA/S) acid/Schiff reactions with unsaturated lipids remains uncertain, the tests have proved useful for the identification of unsaturated lipids provided that the lipids are also sudanophilic and that the positive PFA/S or PAA/S reaction is prevented by prior bromination. Details of the test are given by Lillie (1952, 1954) who also describes the preparation of the per-acids by Greenspan's (1946) method.

The mechanism of the positive PFA/S and PAA/S reactions given by hair is even more obscure. Pearse (1951, 1953) attributed it to disulphide groups, especially of cystine, yielding sulphinic acids which can give positive reactions with Schiff's reagent. Lillie and Bangle (1954) deny that the positive reaction is due to any partial oxidation product of cystine or to adsorbed per-acid. These authors claim the reaction is due to 'an unsaturated insoluble complex of probably acidic nature, whose fatty character

may be suspected but cannot be conclusively demonstrated at the present time'.

In spite of their rather uncertain status as selective oxidants, the organic per-acids warrant further investigation as histochemical reagents, especially for the study of unsaturated lipids. The nature of the oxidation product responsible for the positive Schiff's reactions needs to be established. If it is an aldehyde, there is a risk that it might undergo further oxidation (Swern, 1949). That α-glycols are at least sometimes formed during the PFA/S test when applied to unsaturated lipids is suggested by occasional intensification of the reaction with Schiff's reagent if the tissue section is first treated with periodic acid following oxidation with performic acid (unpublished observations). Iodine should not be used for blocking unsaturated lipids, as was attempted by Findlay (1955), because iodo-compounds can be oxidized by organic per-acids (Swern, 1949).

4.24 Chromic acid and potassium permanganate. Chromic acid was introduced by Bauer (1933) as a selective oxidizing agent. It apparently oxidizes some but not all α-glycols to aldehydes that can react with Schiff's reagent. This gives a somewhat selective demonstration of certain carbohydrates (page 96). Chromic acid, however, has the disadvantage that it can oxidize the initially formed aldehydes to acids that do not react with Schiff's reagent. Such overoxidation might account for the less intense Schiff's reaction usually noted following chromic acid or permanganate as compared with periodic acid oxidation. It would also account for the disappearance of the Schiff reaction upon prolonged oxidation as noted by Burkl (1953). Chromic acid oxidation can be carried out with 5% aqueous chromium trioxide, usually for 60 minutes at room temperature.

Casella (1942) suggested using potassium permanganate as an oxidizing agent followed by Schiff's reagent. The permanganate oxidizes ethylenic groups (Lillie, 1952) as well as α-glycols to aldehydes. Prolonged treatment with permanganate must be avoided because the aldehydes are quickly oxidized to acids. A one percent aqueous solution of potassium permanganate can be used at room temperature for 20 minutes. Permanganate oxidation requires careful standardization before it can be used confidently in histochemistry. Lillie (1951) compared potassium

permanganate, chromic acid and periodic acid as selective oxidizing agents and suggested that PA/S-positive substances giving weak Bauer or Casella reactions might be those having few reactive groups per molecule.

4.3 Blocking methods

Blocking reactions are used for two purposes in histochemistry. One is to increase the selectivity of a method by blocking or removing certain groups that could give a positive reaction. For example, a positive PA/S reaction persisting after a tissue section has been subjected to deamination is almost certainly not due to any of the amino groups discussed earlier (page 40). The other purpose of blocking reactions in histochemistry is to provide confirmation of the presence of a particular type of grouping. For example, a positive PFA/S reaction given by a lipid suggests unsaturation. If the reaction is prevented by prior bromination, the presence of carbon–carbon double bonds is confirmed.

Two types of blocking reactions are used in histochemical analysis:

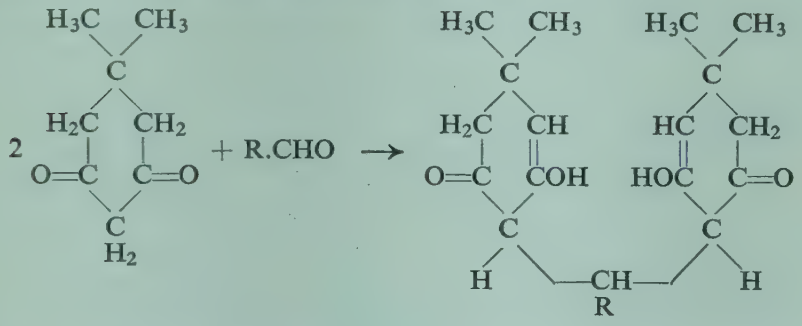
(a) *reversible blockade* where the reactivity of a given group is blocked by esterification or similar treatment but can be restored by appropriate treatment later. Blocking of hydroxyl groups by acetylation (page 49) is an example of this type of reaction.

(b) *irreversible blockade* where the particular group is destroyed. An example of this type is deamination (page 50).

Generally, failure of a test following blockade, although the test was positive before, can be taken as evidence of the groups blocked by the particular method. Persistence of the test, however, does not unequivocally exclude the groups in question. The blockade might have failed or have been reversed by the subsequent test.

4.31 Blocking aldehyde groups. Many reagents have been suggested for blocking aldehyde groups. The reagents vary in the ease with which the blocking can be effected and whether or not it can be reversed. Bisulphite or sulphite is sometimes used but blockade of aldehydes by these agents is readily reversed by prolonged immersion in Schiff's reagent or by mild oxidation—they are quite unsuitable to block free aldehydes before the PA/S test.

Although Pearse (1953) reports some difficulties with dimedone (5,5′-dimethyl-*cyclo*-hexane-1,3-dione), excellent results can be obtained with this blocking agent, using a saturated solution in 1N. acetic acid for as long as 3 days at room temperature or 20 hours at 56° C. The reagent condenses specifically with aldehydes, the internal anhydride of the condensation product being formed in acetic acid solutions (Wild, 1947):

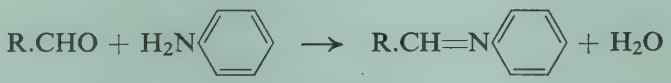


Hydroxylamine (Danielli, 1949) effectively blocks aldehydes. The condensation occurs optimally near pH 4·5–4·7. A suitable

$$R.CHO + H_2NOH \longrightarrow R.CH{=}NOH + H_2O$$

reagent can be prepared by dissolving 10 gms. hydroxylamine hydrochloride and 20 gms. sodium acetate in 40 ml. distilled water. Blocking of aldehydes is usually complete within 1–3 hours at room temperature.

Aniline hydrochloride is often used to block aldehydes. The reagent can be prepared by mixing 9 ml. of redistilled aniline with 8 ml. of concentrated hydrochloric acid and diluting the mixture

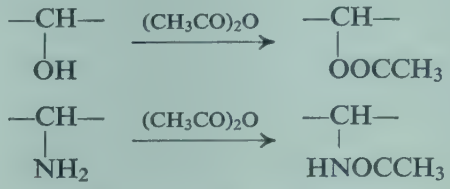


to 100 ml. with distilled water. Treatment of the section for 1–6 hours at room temperature is usually sufficient (Lillie, 1954).

Cyanide is also effective in blocking aldehydes. The blockade can be reversed by periodic acid oxidation (Lillie, 1956).

4.32 Blocking of amino and hydroxyl groups by esterification. Hydroxyl groups can be reversibly blocked by esterification so

that, for example, α-glycols no longer give a positive PA/S reaction. Acetylation of the amino groups in amino compounds oxidized by periodic acid does not block the reaction if it is a primary



amino group but does block the reaction if it is a secondary amino group (Jackson, 1944).

Either acetylation or benzoylation can be carried out on tissue sections. For acetylation (Monné & Slautterback, 1950; McManus & Cason, 1950) 50 percent acetic anhydride in pyridine is usually effective within 6 hours at 56° C. or 24 hours at room temperature. A mixture of 40 percent acetic anhydride in glacial acetic acid containing 1 or 2 drops of concentrated sulphuric acid per 100 ml. can be used similarly but acetylation usually takes place more rapidly. Material giving, for example, a positive PA/S reaction before but not after acetylation can be presumed to contain appropriate hydroxyl or amino groups (page 40). Failure of blockade does not rule out such groups because some tissue constituents are remarkably resistant to the blocking reagents.

If it is desired to reverse blockade due to esterification, the section can be saponified with dilute alkali:

McManus and Cason (1950) treat sections with 0·1N. potassium hydroxide for 45 minutes at room temperature. The reagent is rather drastic so that a shorter time should be used if possible, often 10 minutes is sufficient. Alternatively, ammonium hydroxide can be used as recommended by Lillie (1954): incubate for 24 hours at 37° C. in a mixture of 10 ml. water, 20 ml. concentrated ammonium hydroxide and 70 ml. ethanol in a stoppered container.

4.33 Deamination. Deamination under histochemical conditions by Van Slyke's (1911, 1912) nitrous acid reagent was introduced

by Monné and Slautterback (1950). Tissue sections should be treated for 1–12 hours at room temperature with a mixture of

$$\begin{array}{c}-\mathrm{CH}- \\ | \\ \mathrm{NH_2}\end{array} + \mathrm{HNO_2} \longrightarrow \begin{array}{c}-\mathrm{CH}- \\ | \\ \mathrm{OH}\end{array} + \mathrm{N_2} + \mathrm{H_2O}$$

10 ml. 60 percent aqueous sodium nitrite, 5 ml. glacial acetic acid and 25 ml. water. Such treatment destroys the acidophilia of tissues but usually does not appreciably affect their PA/S reactivity because it does not affect hydroxyl groups.

4.34 Blocking carboxylic and other acidic groups. Free carboxylic and other acidic groups can be blocked by methylation. The

$$\mathrm{R.COOH} + \mathrm{CH_3OH} \longrightarrow \mathrm{R.COOCH_3} + \mathrm{H_2O}$$

method has been used by Fisher and Lillie (1954) in their studies of basiphilia. Methylation can be carried out with methanol containing a small amount of hydrochloric acid (0·5–1·0 ml. concentrated HCl per 100 ml. methanol). Usually, treatment of the tissue section with this mixture for 6 hours at 56° C. or 24 hours at room temperature is adequate.

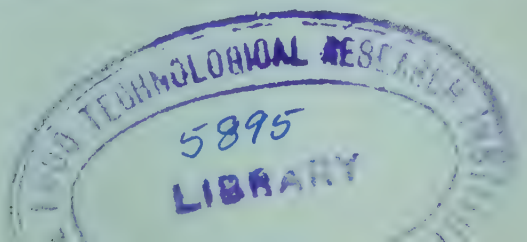
4.35 Blocking ethylenic groups. Ethylenic groups can be most readily blocked by bromination:

$$-\mathrm{HC{:}CH}- + \mathrm{Br_2} \longrightarrow \begin{array}{c}\mathrm{H\ \ H} \\ -\mathrm{C}-\mathrm{C}- \\ \mathrm{Br\ \ Br}\end{array}$$

For unsaturated substances that are soluble in the fat solvents, a saturated solution of bromine in water should be used, preferably a small excess of bromine should be present. Bromination is usually complete within 1–3 hours at room temperature. With substances insoluble in chloroform, a 2 or 3 percent solution of bromine in chloroform can be used for one hour at room temperature. While removal of the bromine and restoration of the ethylenic linkage might be possible, this is impractical histochemically. As mentioned earlier (page 47), iodination cannot be used to block ethylenic groups before treatment with organic per-acids.

4.4 Selective staining

Dyes have been used extensively in cytology and histology for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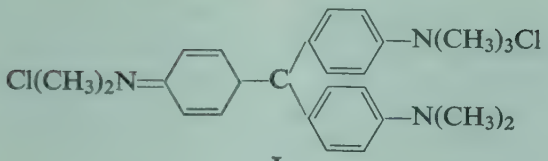
studies of structure but only limitedly in histochemistry for investigations relating to composition. An important limitation of staining methods in histochemical studies has been the lack of exact information on the reactions involved and the factors influencing them. Some of the factors affecting the staining of tissues by acidic and basic dyes, especially reactions between dyes and proteins, include the hydrogen-ion concentration and temperature of the reaction medium, the properties of the dye and the tissue constituents being stained, the nature of the bond between dye and substrate, and the fixation and other preliminary preparation of the tissues. These factors have been discussed in some detail in a review by Singer (1952).

The most extensive use of selective staining methods in histochemistry has been in the study of nucleic acids, using basic dyes (pyronin/methyl green test, page 108), and in the study of acid mucopolysaccharides, using metachromatic dyes (page 103). Acidic and basic dyes have found limited use in studies on proteins.

4.41 Basic dyes. Basic dyes are used in studies on nucleic acids and, less often, on proteins. Methyl green (I) is the dye generally used for deoxyribonucleic acids, pyronin (II) for ribonucleic acids and methylene blue (III) for proteins. The staining of nucleic acids by basic dyes, especially deoxyribonucleic acids by methyl green, has been studied extensively by Kurnick who has recently reviewed these and other investigations (Kurnick, 1955). Swift (1955) has also reviewed the staining of nucleic acids by basic dyes. Einarson (1951) has recommended the chrome alum lake of gallocyanin (IV) for the selective staining of nucleic acids.

Early studies on the staining of proteins and other tissue constituents by buffered solutions of dyes were carried out by Pischinger (1926, 1927) whose methods are still recommended by Lipp (1955). One of the most important investigations was carried out by Levine (1940) who demonstrated that the staining of proteins is influenced by interactions between dye and buffer, and between buffer and protein as well as between dye and protein. Dempsey, Singer and Wislocki (1946; Dempsey & Singer, 1946; Singer & Wislocki, 1948) used methylene blue as the basic dye and orange G as the acidic dye in their studies on the staining of proteins. The staining of nucleic acids has been reviewed by Singer (1954) in his



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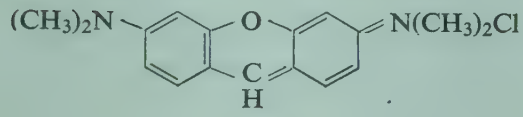

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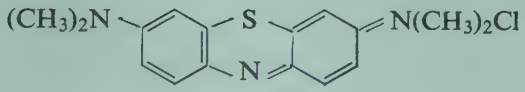

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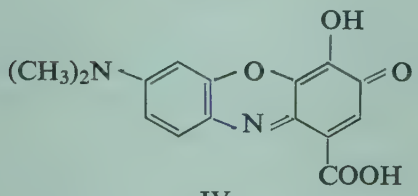
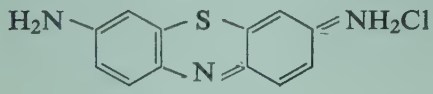


IV

contribution to the Histochemical Society's symposium on 'basophilic components of cytoplasm'.

4.42 Metachromatic dyes. Certain metachromatic dyes, especially thionin (I) and the closely related azure A (II) and toluidine blue



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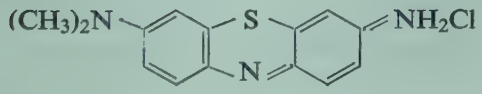

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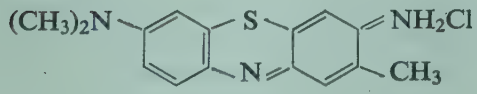


III

(III), are used in histochemistry. The mechanism and significance of metachromatic staining have been studied by many investigators. Lately, the trend has been to study dye-substrate reactions as isolated systems using pure substances. Such studies are important in providing exact information regarding the reactions involved but the limitations imposed by the use of pure substances (page 21) must not be overlooked. Recent histochemical and physico-chemical studies of this type have been reviewed by Schubert and Hamerman (1956). They conclude that certain high molecular weight substances with free anionic groups can stain metachromatically, including acid mucopolysaccharides, nucleic acids, and some acidic lipids that can polymerize into micelles of high molecular weight. Such substances containing ester sulphate groups stain more intensely than those containing carboxylic or phosphate groups.

Sylvén (1954) found that metachromatic staining depends upon the surface density of the electronegative charges on the chromotrope, the orientation of the molecules of dye that have reacted with it, and the presence of water. The charge density is the important property of the chromotrope influencing its reaction with a metachromatic dye. The distribution of electronegative charges on the surface of the substrate determines the alignment of the dye molecules and the degree of metachromasia. A distance between anionic groups of about 5 Å. appears to be necessary for metachromatic staining. The intensity increases as the distance between adjacent dye molecules attached to the surface becomes less than 5 Å. It also increases with increasingly orderly alignment of the dye molecules which is favoured by the presence of both hydrophilic and hydrophobic groups on the dye molecule.

The reaction between dye and chromotrope is particularly influenced by salts and proteins (Schubert & Hammerman, 1956). With low concentrations of the reactants, even low salt concentrations inhibit the metachromasia. The inhibition is due to the salt cations, increasing with their valence, and probably depending upon competition between the salt and dye cations for anionic sites on the chromotrope. Such inhibitory effects are seldom of importance in histochemical studies because the salts are washed out of the tissue sections and relatively high concentrations ($> 10^{-3}$ M) of dyes are usually used. Inhibition of metachromatic staining by proteins is more important. Proteins can act in at least

three ways: competing like inorganic cations, forming covalent bonds with anionic groups, or reacting with polysaccharides to block their anionic groups by steric hindrance. The metachromasia of hyaluronic acid seems to be more susceptible than that of ester sulphate mucopolysaccharides to protein inhibition. Such inhibition can be eliminated sometimes by treatment with proteolytic enzymes.

Whether or not true metachromatic staining should resist alcoholic dehydration has been a much debated question. Lison (1935, 1935*a*, 1953) requires that it resist alcohol. Kramer and Windrum (1955) likewise conclude from their findings that 'when the metachromatic staining reaction is used for histochemical purposes it is essential that steps be taken to differentiate histochemically significant irreversible metachromasia from the non-specific reversible metachromasia which can be exhibited under certain conditions by an extremely wide range of tissue elements. This can be done either by mounting the sections after staining in Apathy's medium or syrup of laevulose, as recommended by Lison (1935, 1936), but far more effectively by alcoholic dehydration.'

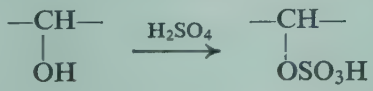
On the basis of their detailed studies on several metachromatic dyes, Kramer and Windrum (1955) recommend the following method which certainly gives good results.

1. Bring paraffin sections to water and stain for 10 minutes in either 0·01% or 0·1% azure A in 30% ethanol. The weaker reagent should be used for strongly metachromatic materials and the more concentrated reagent for weakly metachromatic materials.
2. Rinse in distilled water, then in 70% ethanol.
3. Dehydrate for 2–3 minutes in each of two changes of absolute ethanol.
4. Clear in xylene and mount in neutral balsam or synthetic resin.

4.5 Esterification

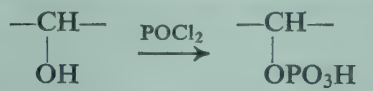
Two esterification methods have been introduced that promise to be of value especially in the study of carbohydrates. Kramer and Windrum (1954) have shown that at least certain tissue carbohydrates can be converted to their sulphuric esters by a variety

of reagents including sulphuric acid, chlorosulphonic acid in pyridine and fuming sulphuric acid in acetic anhydride and ether.



The esters formed by treatment with one of these reagents can be stained with metachromatic dyes (page 55). This combined sulphation and metachromatic staining method should prove to be a valuable research method to be used with the periodic acid/Schiff (page 44) test in studies on carbohydrates (page 94) because the two tests depend on entirely different reaction mechanisms. Neutral polysaccharides, glycoproteins, and simple acid polysaccharides can be rendered metachromatic. The reagents differ in the number and variety of carbohydrates sulphated, glycogen being one of the more difficult to esterify.

Landing and Hall (1956) have adapted the phosphorus oxychloride method of Lohmar, Sloan and Rist (1950) to the phosphorylation of tissue sections. Various tissue carbohydrates, including mucins, glycogen and some connective tissue components, as well as certain lipids and proteins apparently can be phosphorylated in this way. Just as after sulphation, some previously



non-metachromatic materials stain metachromatically, while certain previously metachromatic substances lose some of their metachromasia following phosphorylation. However, the results obtained with sulphation and with phosphorylation are not identical.

4.6 Selective extraction

The histochemical identification of a given constituent is sometimes facilitated or confirmed by studying its selective removal by chemical reagents or enzymes, in much the same way that blocking reactions aid identification in other cases. Presently available extraction methods, like the blocking methods, are far from infallible. Failure to extract a constituent is not unequivocal proof of its absence. In addition, the extraction methods are seldom

entirely specific. Despite such limitations, these methods can be of considerable assistance in histochemical analysis.

4.61 Chemical reagents. The principal non-enzymic extraction methods are the selective extraction of lipids by organic solvents (page 76) and the selective removal of nucleic acids by hydrolysis with certain acids (page 120). By definition, the lipids are soluble in 'fat solvents'. The solubility of purified lipids in pure solvents is of importance in the chemical identification of the lipids. In tissues, however, lipids are rarely, if ever, present in the pure state. Usually they occur as mixtures of lipids dissolved in one another. Under such conditions the rules of solubility in the identification of lipids do not necessarily hold. The total or differential extraction of lipids from tissues is possibly complicated by at least three additional factors (Lovern, 1955):

(*a*) some lipids could be combined with proteins or carbohydrates,

(*b*) some lipids are soluble in only a limited range of solvents, and

(*c*) some 'fat solvents' also extract other tissue constituents that are not lipids.

If the extraction is carried out on fixed tissues, additional sources of error are introduced. Oxidative fixing agents such as chromic acid greatly alter the physical and chemical properties of lipids even rendering some insoluble in lipid solvents. Even fixation in formaldehyde has been observed to alter the solubility of compound lipids in tissues and to alter the sudanophilia and optical properties of some lipids (Lison, 1953). It is for such reasons, that extraction of fresh tissues is recommended in Chapter 5 (page 76).

It is often desirable to remove one or both nucleic acids from tissue sections; for example, in studies with ultraviolet light absorption which does not distinguish between deoxyribonucleic acids and ribonucleic acids. Similarly, control slides from which the nucleic acid under investigation has been removed can be compared with test slides to establish the specificity of the method being used. Various acids have been suggested for the selective or complete removal of the nucleic acids from tissue sections. Their use is discussed in Chapter 7 (page 123).

4.62 Enzymic hydrolysis. The principle underlying the use of enzymes as reagents in histochemistry is that by treatment of a microscopical preparation with a highly purified enzyme will remove those components of the cell or tissue that are composed of the specific substrate for the enzyme. This presupposes that the enzyme preparation really is pure and specific in action and that the enzyme has access to the substrate. At present, there is no way of establishing the specificity of an enzyme for one substrate. Moreover, many of the enzyme preparations used in histochemistry are impure and consist of more than one type of enzyme (Pirie, 1940). As Danielli (1947) pointed out, it is theoretically possible that a layer of protein over a nucleic acid would prevent the specific nuclease gaining access to its substrate; however, the frequently successful use of enzymes in histochemical studies would suggest this does not occur too often.

Lillie (1954) has outlined three general principles underlying the use of enzymes in histochemical studies and the interpretation of the results obtained with them:

(*a*) Since enzyme preparations are rarely free of any action other than their specific activity, hydrolysis does not necessarily indicate that the component consists of the specific substrate alone. Examples are provided by some ribonuclease preparations which are also proteolytic and by saliva which, although usually used as a source of amylase, also often contains a ribonuclease (Bradbury 1956). As a corollary, it should be noted that sometimes when a specific component is removed from a given structure, the structure can disintegrate because of the loss of its integrity even though it is not composed solely of the specific substrate of the enzyme.

(*b*) Failure of an enzyme to hydrolyse a given component under conditions previously established as optimum for the action of the enzyme does not conclusively exclude the presence of the substrate. It is possible, for example, that the substance is so combined with other constituents that it is no longer accessible to the enzyme.

(*c*) All enzymic digestion studies must be properly controlled. Additional sections should be treated with the medium for the enzyme or with the solution of the enzyme after suitable inactivation by heat or specific inhibitor.

The effective use of enzymes as selective reagents in histochemical analysis requires careful control of all the factors influencing the action of the enzyme as well as those affecting the test used to detect the substrate (Kaufmann, Gay & McDonald, 1950). In so far as the enzyme reaction is concerned, the most important factors are the purity and concentration of the enzyme, the composition of the medium including pH, concentration of buffer and other salts, and any activators or inhibitors, as well as the time and temperature of incubation and the stability of the enzyme under the conditions of the test.

Fixation affects the entire test, both the action of the enzyme and the subsequent test. The effects of fixation begin with the preservation of the substrate whose quantity and distribution can be affected. Fixation also affects the action of the enzyme and, in some cases, can interfere either by blocking the substrate so that the enzyme cannot gain access to it or by being retained in the tissues and acting as an inhibitor of the enzyme. Another possibility that should not be overlooked is that the fixing reagent might so alter the substrate that although it is hydrolysed by the enzyme, the hydrolysis products cannot diffuse away.

Other aspects of the use of enzymes as selective reagents are discussed by Glick (1949) and by Pearse (1953). Benditt and French (1953) have emphasized the need for caution when using relatively impure enzymes, especially in interpreting the results obtained in studies on polysaccharides. Their investigations were carried out on cartilage, using testicular (hyaluronidase) and malt (amylase) extracts and crystalline trypsin, chymotrypsin and pepsin.

Of the available, more or less purified enzymes, the nucleases (pages 120 and 121), especially ribonuclease, have been used most extensively in histochemistry. Amylases (page 100) and hyaluronidases (page 97) have found certain applications while the proteolytic enzymes (page 134) have been used infrequently.

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CHAPTER 5

Lipids

5.11 Definition. In histochemistry as in biochemistry, 'there is probably no field in which the terminology is less uniform or more arbitrary than that employed by investigators of lipids' (Lovern, 1955). The lipids can be described as a group of substances that are generally soluble in certain 'fat solvents' but not water and that include the higher fatty acids, their naturally occurring compounds and substances found naturally in association with them (Bloor, 1943). However, even this description presents problems of delineation and interpretation (Lovern, 1955).

5.12 Classification. A classification of histochemically demonstrable lipids based on the above definition is presented in Table 5.1. The *simple lipids* are usually defined as esters of fatty acids with alcohols. Esters with glycerol (triglycerides) or alcohols of higher molecular weight (ester waxes), other than the sterols, have been included under this heading. The *compound lipids* contain additional constituents such as the phosphoric acid and nitrogenous bases in phospholipids or the carbohydrates in glycolipids. The *derived lipids* include some of the constituents of the simple and compound lipids that can be obtained from them, usually by hydrolysis. Because of their histochemical properties, the *sterols and sterol esters* have been placed in a separate category. A group of *associated substances* have been included even though they differ in chemical structure.

5.13 Special problems. The lipids present a number of problems for the histochemist. The individual compounds in any class of lipids have such similar chemical and physical properties that they cannot be distinguished individually *in situ* by available techniques. With few exceptions, the histochemical characteriza-

tion of lipids is limited to determining which classes are represented.

TABLE 5.1

A CLASSIFICATION OF HISTOCHEMICALLY DEMONSTRABLE LIPIDS

SIMPLE LIPIDS
- Triglycerides
- Ester waxes

COMPOUND LIPIDS
- Phospholipids
  - Phosphatidic acids
  - Phosphatidyl esters
  - Inosityl phosphatides
  - Acetal phosphatides
- Glycolipids
  - Cerebrosides
  - Gangliosides

DERIVED LIPIDS
- Fatty acids
- Fatty aldehydes
- Alcohols
- Nitrogenous bases
- Autoxidation products

STEROLS AND STEROL ESTERS
- Cholesterol
- Cholesteryl esters

ASSOCIATED SUBSTANCES
- Carotenoids
- Fat-soluble vitamins
- Steroids
- Lipid pigments

Lipids almost always occur as mixtures, usually of compounds in several classes. In such mixtures the individual constituents no longer necessarily exhibit their characteristic properties, especially physical properties such as solubility and melting point. Consequently, it is not always possible to establish the presence of certain classes by histochemical methods depending upon these properties, for example, differential extraction (see pages 56 and 76).

Lipids can exist in cells and tissues under various conditions which influence the demonstrability of the lipids. Cain (1950) has described four possibilities:

(*a*) lipids detectable as such in living or fixed tissues,

(*b*) lipids present as such in living tissues but detectable only after fixation,

(*c*) lipids not present as such in living tissues but demonstrable after fixation,

(*d*) lipids that can be detected only after special treatment ('unmasking') in addition to fixation.

5.14 Identification. The differential histochemical properties of the various classes of lipids are summarized in Table 5.2. Unstained frozen sections as well as adjacent sections treated with one of the oil soluble colorants (page 68) should be studied to determine whether demonstrable lipids are present. Initial subdivision into acidic and non-acidic lipids can be made with the Nile blue test. Further differentiation depends upon the special tests described for the various classes.

5.2 Tissue preparation

Generally, the preparation of tissues for studies on lipids presents no special difficulties. It is important, of course, that the tissues are not exposed to fat solvents at any time.

5.21 Fixing. Formaldehyde is the most suitable fixing agent. Acidic impurities in formalin (34–40% aqueous solution of formaldehyde) can be removed with an anion exchange resin (for example, Amberlite IR-45, Rohm and Haas Co., Philadelphia, U.S.A.; C. Lennig and Co., London, W.C.1) or neutralized with calcium carbonate or magnesium oxide.

Baker's (1944) mixture of 10% formalin containing 1% calcium chloride can be recommended for fixing tissues for studies on most lipids. Cobalt chloride or cobalt nitrate can be added (McManus, 1946) to render phospholipids less soluble and to convert fatty acids to soaps. Lillie (1954) has suggested using 2% calcium acetate instead of 1% calcium chloride because of the buffering action of the acetate. With acetates, however, there is a risk of destroying some cytoplasmic inclusions. As Gomori (1952) has pointed out, the alleged loss of lipids (as estimated chemically) during prolonged storage in formaldehyde requires accurate verification. No appreciable histochemical changes have been observed in mildly or severely fatty livers after 6 years' storage in formaldehyde-calcium (Casselman & George, unpublished).

Other fixative mixtures can be used provided they do not contain fat solvents. Mixtures containing oxidizing agents such as chromic acid dichromates or mercuric chloride should be avoided except when required as part of a test. The physical and chemical properties of some lipids are progressively modified upon exposure to such agents (Casselman, 1951). For some methods such as the plasmal test, fresh tissues might be preferable.

TABLE 5.2

HISTOCHEMICAL DIFFERENTIATION OF LIPIDS

		Compound Lipids			*Derived Lipids*		
Test	*Simple Lipids*	*Phospho-lipids*	*Acetal-phostd.*	*Glyco-lipids*	*Fatty Acids*	*Choles-terol*	*Choles. Esters*
Sudan black B	+	+	+	+	+	(+)	(+)
Nile blue	N	A		N	A	(N)	(N)
Acid haematein	0	+		0	0	0	0
Plasmal	0		+				
Periodic acid/Schiff	0	0		+	0	0	0
Schultz	0	0		0	0	+	+
Digitonin						+	0

+ = positive reaction
0 = negative reaction
N = non-acidic
A = acidic
( ) = reaction not always obtained
blank = test not ordinarily used

5.22 Embedding. Frozen sections for studies of lipids can be cut from many tissues without prior embedding. In some cases, especially with small pieces or fragile tissues, embedding in gelatin is necessary. If sections must be cut on a paraffin-type microtome, the tissues can be embedded in polyethylene glycols (Carbowax) but neither paraffin nor celloidin can be used (page 28).

5.23 Sectioning. An important, and rather frequent, technical artifact in frozen sections of tissues containing large amounts of free lipids is that due to dislocation of fat by the microtome knife. As a result, there are droplets of fat lying on otherwise fat-free cytoplasm or nuclei or in tissue spaces and possibly around the tissue section, especially if it is embedded in gelatin. Such artifact is sometimes difficult to identify with certainty. Often, it can be recognized by the unusual distribution and shape of the fat droplets and by their being in a plane of focus above and below that of the section.

5.3 General methods

5.31 Microscopy. The histochemical study of lipids should begin with the microscopical examination of an unstained frozen section of fresh or briefly (6 hrs.) fixed tissue. The presence of refractile droplets, granules or crystals suggests the presence of lipids in discreet forms. Diffuse or 'masked' lipids, of course, will not be seen. Preliminary study of an unstained section is important because not all lipids are coloured by the oil-soluble colorants that are ordinarily used for the detection of lipids in general.

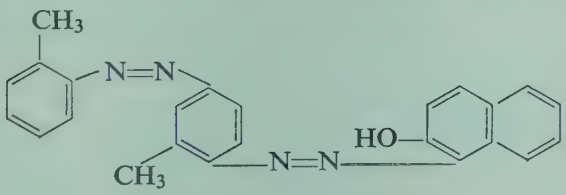
Further investigations can be carried out with polarized light if desired although Lison (1953) has shown that the method is of less value than previously thought. No conclusions can be drawn if the objects are optically inactive or optically active but not birefringent. Birefringence, however, is indicative of spherocrystals. These can be formed by cholesterol and its esters or by the compound lipids but not by the triglycerides or fatty acids.

5.32 The oil-soluble colorants. The oil-soluble colorants provide the most widely used general test for lipids even though not all free lipids can be demonstrated, those having too high melting points are not coloured. Various reagents have been recommended. Among the first were the Sudan dyes. Their use led to

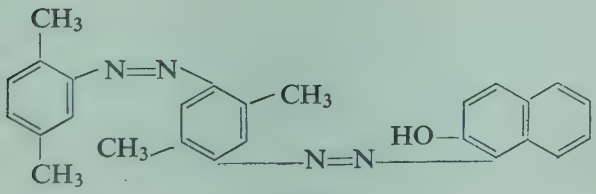
the term, *sudanophilia*, designating the property of being colourable by such reagents.

5.321 Mechanism of coloration. The coloration of lipids by an oil-soluble colorant depends upon the reagent dissolving in the lipids (Michaelis, 1901, 1910; Cain, 1950). This, in turn, depends upon the physical state of the lipids, and therefore, their melting points, and upon the partition coefficient of the reagent between its solvent and the lipids. The properties of the tissue lipids vary widely. At the one extreme are the solid lipids, with high melting points, in which little, if any, of the reagent can dissolve. At the other extreme are the oily lipids, having low melting points, in which the reagent dissolves readily. With the formation of lipid mixtures, any resultant decrease in melting point will enhance the sudanophilia. On the other hand, a thin 'shell' of hard lipid could conceivably prevent the coloration of an otherwise sudanophilic droplet. Lipids that have high melting points and are not coloured under ordinary conditions can be coloured if the reagent is heated to near or above the melting points of the lipids.

The remarkable solubility of the oil-soluble colorants in lipids is related to a lack of acidic or basic properties (Michaelis, 1901). Most of the oil-soluble colorants are azo dyes, usually azo-β-naphthols (Conn, 1953). Such dyes with a β-hydroxyl group undergo rearrangement to quinoid forms that are essentially neither acidic nor basic. The structures of Sudan IV (I) and the closely related, more intensely coloured oil red O (II) are given

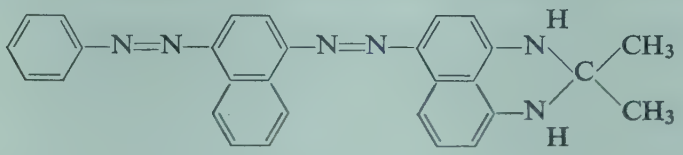


I

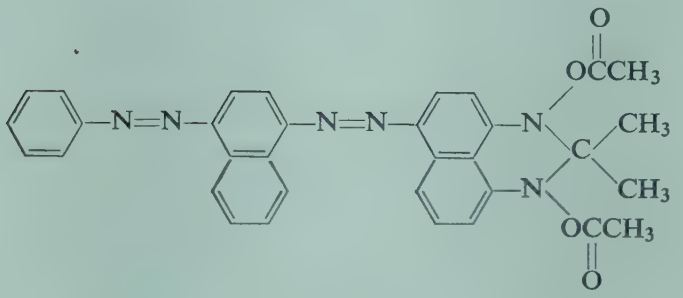


II

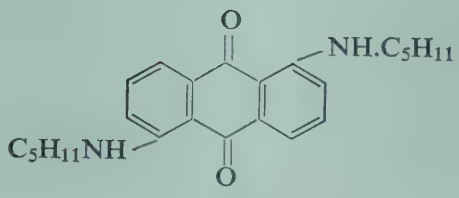
below. Sudan black B (III) is unusual. It is an azo dye but lacks the typical β-hydroxyl group. Furthermore, it contains two secondary amino groups, that make it a slightly basic dye. This property might be responsible for the excellent staining of phospholipids obtained with this reagent. However, the basic property also leads to some staining of non-lipid substances. For this reason, Lillie and Burtner (1953; Casselman, 1954) introduced the acetylated derivative (IV) and ethanol extraction (see



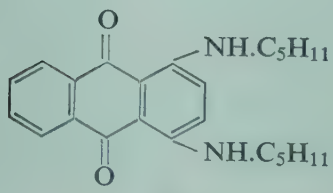
III



IV



V



VI

page 73). A few of the oil-soluble colorants, such as coccinel red (V) and oil blue NA (VI), are amylaminoanthraquinones.

5.322 Choice of reagent. For some time after its introduction by Daddi (1896), Sudan III was the only important lipid colorant. Later, Sudan IV was recommended by Michaelis (1901) and gradually replaced Sudan III because of its deeper colour and more intense coloration of lipids. For the same reasons oil red O was introduced by French (1926) and recommended as one of the best by Lillie (1944). For most lipid studies, Sudan IV or, preferably, oil red O gives excellent results especially when the studies are limited to all but the smallest lipid droplets and lipid-containing intracellular structures such as mitochondria. Unquestionably the most sensitive lipid colorant is Sudan black B.

5.323 Choice of solvent. The oil-soluble colorants are frequently used in alcoholic solutions, 70% ethyl alcohol (*e.g.* Baker, 1956) and 60% isopropyl alcohol (Lillie & Ashburn, 1943) being the most popular. These solvents afford rapid staining but might remove some lipid. With more dilute alcohols, there is less risk of extracting lipids but also less of the reagent can be dissolved so that the staining time must be increased and lipid coloration is less intense. Owing to the volatility of the solvent, alcoholic solutions tend to precipitate during storage and even during use.

Ethylene and propylene glycols have been recommended (Hartman, 1940; Chiffelle & Putt, 1951) as giving stable solutions and good coloration. Propylene glycol certainly has proven satisfactory as a solvent for oil red O or Sudan black B. However, Gomori (1952) has cautioned that propylene glycol has appreciable solvent power for water-insoluble organic compounds.

Gomori (1952) recommended 60% triethylphosphate. This solvent has no effect upon lipids. Solutions of oil-soluble colorants in it have practically the same colouring power as alcoholic solutions and show no tendency to precipitate. The results obtained with them are excellent.

5.324 Preparation of reagent solutions. The following procedures for preparing solutions of the reagents can be used for oil red O, Sudan black B, or any other oil-soluble colorant.

70% Ethanol: Add about 1 gm. of the reagent to 100 ml. of 70% ethanol in a boiling flask with a reflux condenser, preferably

attached by a ground glass joint. Heat gently and boil for 3 minutes. Filter the hot mixture through fluted filter paper and cool. Before use the solution should be filtered again.

Isopropyl Alcohol: Prepare a saturated stock solution by adding about 1 gm. of the reagent to 100 ml. 98% isopropyl alcohol. Saturation can be achieved more rapidly if the mixture is warmed, for example, in the paraffin oven. Allow the excess reagent to settle to the bottom of the stock bottle.

For use, add 20 ml. distilled water *to* 40 ml. of the stock solution. Mix well. After 10 minutes, filter the supersaturated solution through fluted filter paper or a coarse, sintered glass filter. The filtrate can be used for a few hours but should be discarded when precipitation of the reagent commences.

Triethyl Phosphate: Add about 1 gm. of the reagent to 100 ml. of 60% aqueous triethyl phosphate. Either heat the mixture to 95–100° C. for 5 minutes, stirring continually, or place it in the paraffin oven for 2 or 3 hours, stirring occasionally. Filter the hot mixture through fluted filter paper and cool. Filter again before using.

5.325 Coloration procedures. The procedures for using the above solutions of the oil-soluble colorants are essentially the same, differing only in certain details as shown below.

1. Bring the section to 50% ethanol, 60% isopropyl alcohol or 60% triethyl phosphate, depending upon what solvent has been chosen for the reagent. With either alcohol, it is advisable to limit the time to 20–30 seconds so that no lipids are extracted or dispersed. Sections can be left safely for longer in the triethyl phosphate. By replacing water with the particular solvent, this preliminary treatment helps to prevent precipitation of the oil-soluble colorant due to dilution in the next step.
2. Immerse in the reagent solution long enough to give adequate coloration of lipids. Usually, from 2 to 5 minutes is sufficient for Sudan black B, but up to 15 minutes might be necessary with the red dyes. The reagents can be used at room temperature, at 37° C. or at 56° C. When either alcoholic solution is used at the higher temperatures the staining jar must be tightly stoppered to prevent loss of

solvent and consequent precipitation of the reagent. Triethyl phosphate does not present this problem.

3. Wash off the excess reagent using some of the diluted solvent at the same temperature as the reagent. With either ethyl or isopropyl alcohol, the time must be as short as possible, preferably not over 5–10 seconds. With longer exposure to either alcohol, especially if warm, some of the colorant will be extracted from the lipids (see below regarding extraction by solvents).
4. Bring to water and counterstain nuclei. With Sudan black B, either neutral red or Mayer's carmalum is recommended. With the red colorants, one of the haematoxylins, such as Gomori's or Harris's, can be used.
5. Counterstain the cytoplasm if desired. With Sudan black B, this is unnecessary. Light green SFS gives good contrast with the red colorants but there is a risk of obscuring very fine lipid droplets unless the counterstain is kept very light.
6. Mount in Farrant's or a similar medium.

5.326 Non-specific coloration. The oil-soluble colorants are generally regarded as specific reagents for lipids. Actually, they are only highly selective reagents. They are not completely inert chemically, but can act as weakly basic dyes staining some tissue elements that probably contain no lipid. Such staining usually occurs more slowly than coloration of lipids and is more apt to take place when the reagents are used at higher temperatures.

Non-specific staining can be distinguished from true coloration of lipids by *decoloration.* Since the coloration of lipids by an oil-soluble colorant depends upon the reagent dissolving in the lipids, it follows that the reagent should also be extractable from the lipids by an excess of a suitable solvent. This is the basis of a test of the presumed lipid nature of sudanophilac material used by Lillie and Burtner (1953) in their studies on the stable sudanophilia of leucocyte granules. Various solvents can be used, including 60–100% ethanol, acetone, xylene, and diethylene and dipropylene glycols, provided the solvent does not extract the sudanophilic tissue constituent. Oil red O, for example, is completely removed from adrenal lipids within 3 hours by ethylene glycol which does not extract the lipids. 85% ethanol has proved useful with various substances insoluble in it while acetone and

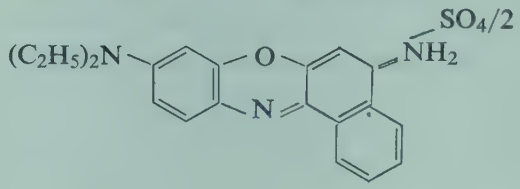
xylene rapidly extract the colorants from insoluble lipid pigments such as ceroid. It is essential that recoloration be attempted following extraction of the colorant. Material that gives a true sudanophilic reaction can be decoloured and recolored repeatedly provided it is not extracted by the decolorizing reagent.

Non-specific staining can usually be avoided by using an *esterified reagent*, acetylated derivatives being the most readily prepared (Lillie & Burtner, 1953; Casselman, 1954). The acetylated reagents colour lipids almost as intensely as the unesterified colorants, often giving greater contrast between lipid droplets and surrounding cytoplasm. Sometimes during acetylation or even storage of an alcoholic solution, Sudan black B undergoes degradation to products that are not lipid colorants but may or may not act as basic dyes.

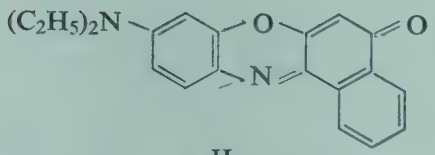
5.4 Differential methods

*5.41 Nile blue test** (*acidic and non-acidic lipids*). One of the first steps in the analysis of sudanophilic lipids is to distinguish between acidic and non-acidic lipids using the Nile blue test. The 'acidic' lipids include fatty acids and phospholipids. The 'non-acidic' lipids include the other classes. The term 'neutral lipids' is not recommended for these because it is usually synonymous with triglycerides.

Nile blue (C.I. No. 913) was introduced as a histochemical reagent by Smith (1908), who reported the simultaneous blue coloration of fatty acids by the oxazine (I) component of this



I



II

* See Addenda on page 180.

reagent and the red coloration of neutral fats by its oxazone (II) (Thorpe, 1907). Although the specificity of the test was questioned by Kaufmann and Lehmann (1926), Cain (1947, 1948) was able to confirm and extend Smith's observations. Lennert (1955) also found that provided they are liquid or greasy at the staining temperature, fatty acids are coloured blue while their glyceryl or other esters are coloured red or pink and higher alcohols, purplish red.

Cain (1947, 1948) concluded that Nile blue distinguishes between acidic and non-acidic lipids in general. The red oxazone colours the non-acidic lipids by the same mechanism as the other oil-soluble colorants. Cain (1947, 1948) believed that the oxazine simply reacts with the acidic lipids to form blue salts. Lillie (1956) has shown that two processes are involved. The first is considered to be 'an oil solubility phenomenon in which the deep blue colour is determined by indicator properties of the dye'. This reaction occurs promptly even with very dilute, strongly acidic, aqueous solutions of Nile blue. The colour is also promptly extracted with acetone or ethanol. The second process is considered to be salt formation, similar to that taking place with other basic dyes, resulting in lighter, greener staining. This reaction occurs more slowly and requires a more concentrated, less acidic reagent. The colour is resistant to acetone and relatively resistant to ethanol. Both processes are prevented by methylation of the lipid and are attributed to carboxylic groups.

Because the oxazine component is a basic dye it can form blue salts not only with acidic lipids but also with other basiphilic materials. Nuclei, for example, are well stained with Nile blue. Consequently the results obtained with this reagent must be compared with those obtained with one of the oil-soluble colorants to establish what substances stained by Nile blue are also sudanophilic and, therefore, lipids.

5.42 Performic acid/Schiff test (*unsaturated lipids*). A variety of tests have been suggested for the detection of unsaturated lipids. Most of them are non-specific and relatively unselective. Osmium tetroxide was introduced by Starke in 1895. Cain (1950) has specified precise conditions under which the test can be carried out. Even so, osmium tetroxide cannot be highly recommended as even a selective test for unsaturated lipids.

For the demonstration of unsaturated lipids, selective oxidation by an organic per-acid followed by Schiff's reagent is probably the most suitable test now available. As discussed in Chapter 4, this test apparently depends upon oxidation of ethylenic linkages to aldehydes that can be demonstrated by Schiff's reagent (page 45). Performic acid is most commonly used. Material that has been identified as lipid by previous tests and that gives a positive reaction with the performic acid/Schiff test can be considered to contain one or more double bonds. Their presence can be confirmed by blocking the reaction by prior bromination using bromine water (page 51).

5.43 Selective extraction. Selective extraction by organic solvents is a procedure that can be as misleading some times as it is helpful at other times in the characterization of lipids. While certain classes of lipids have characteristic solubilities when in the pure state, these characteristics are often lost when these lipids are mixed with other lipids or associated with other tissue constituents such as proteins or carbohydrates.

Extractions with hot solvents can be carried out in various ways. Ideally, a semi-micro Soxhlet apparatus should be used so that the extraction is by warm, pure solvent. Alternatively, a small boiling flask and reflux condenser (Pearse, 1951, 1953) or a beaker with Florence-flask 'condenser' (Lillie, 1954) can be used. An electric light bulb of suitable wattage provides a convenient and safe source of heat. Extractions can also be carried out in well stoppered tubes or jars placed in the paraffin oven.

For the removal of all lipids, a hot mixture of equal parts of chloroform and methanol has been used extensively while hot pyridine gives the most complete extraction of lipids (Baker, 1946).

Keilig (1944; Pearse, 1949, 1953) introduced a scheme of selective extraction of fresh tissues that should be studied further. It can give good results (*e.g.* Shafig & Casselman, 1954; Casselman & Baker, 1954) when used in conjunction with Sudan black B and the Nile blue and PA/S tests. The solvents and the classes of lipids that should be extracted are given in Table 5.3. Morphologically, the results are not so good as might be desired. During extraction of the tissue, those lipids that are insoluble in the reagent, while not removed, are sometimes dispersed to some

TABLE 5.3

DIFFERENTIAL EXTRACTION OF LIPIDS

Solvent	*Simple and Derived Lipids*	*Compound Lipids*	
		Phospho-lipids	*Glyco-lipids*
Cold acetone	E	I	I
Hot acetone	E	I	E
Hot ether	E	E	E

E = extracted I = insoluble

extent or displaced to one side of the cell. This is not too serious a problem because the purpose of the extracted specimen is to aid in the chemical identification of the lipid. The localization of the lipid can usually be determined from unextracted preparations. It should be noted that the properties of tissue proteins can be altered by the extraction, sometimes resulting in changes in histochemical reactivity, stainability, or solubility.

Adding cadmium nitrate to cold acetone helps to prevent dislocation or loss of the compound lipids through emulsification (Ciaccio, 1934). Fixation of the tissues in formaldehyde-calcium also reduces the tendency for phospholipids to become emulsified during extraction with hot or cold acetone.

Proteins tend to prevent the extraction of certain lipids especially after formaldehyde fixation. An example of this is provided by the cerebrosides (kerasin) in Gaucher cells. These compound lipids can be extracted readily from fresh tissue using hot methanol and chloroform but are almost unextractable after fixation (Morrison & Hack, 1949).

5.5 Simple lipids

The simple lipids include the triglycerides and ester waxes. Of all the classes of lipids, the simple lipids can be identified least satisfactorily by histochemical tests. There is no positive test for the simple lipids. Their presence can be presumed only by exclusion

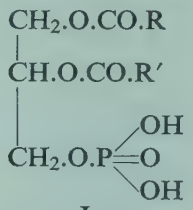
$$\begin{array}{l} CH_2.O.CO.R \\ | \\ CH.O.CO.R' \\ | \\ CH_2.O.CO.R'' \end{array}$$

(Triglyceride)

of other classes. If there is refractile, sudanophil material that is non-acidic and that does not give positive reactions for any of the compound or derived lipids or associated substances, it can be reasonably assumed to be triglyceride.

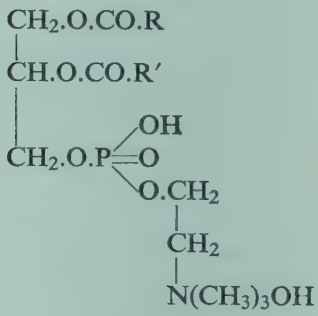
5.6 Compound lipids

5.61 Phospholipids. The least complex of the phospholipids are the phosphatidic acids (I). These acids are closely related to the



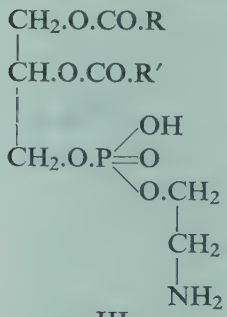
I

(Phosphatidic acid)



II

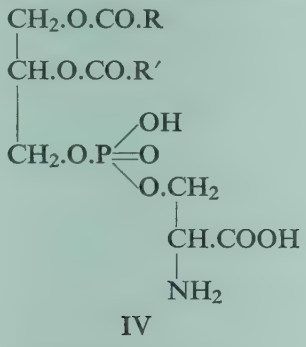
(Phosphatidyl choline)



III

(Phosphatidyl ethanolamine)

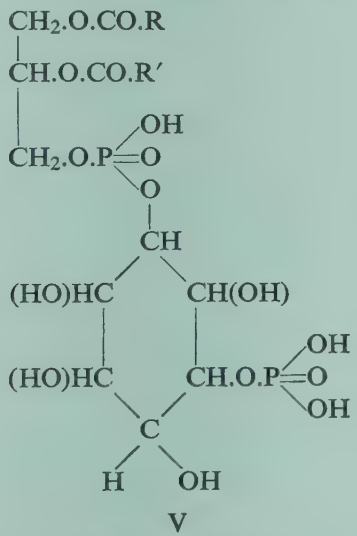
triglycerides differing in that one fatty acid (probably in the α-position) is replaced by phosphoric acid. Three types of phospholipids are esters of the phosphatidic acids and a nitrogenous alcohol: phosphatidyl choline (II, lecithin), phosphatidyl ethanolamine (III, cephalin), and phosphatidyl serine (IV). The inositol



IV

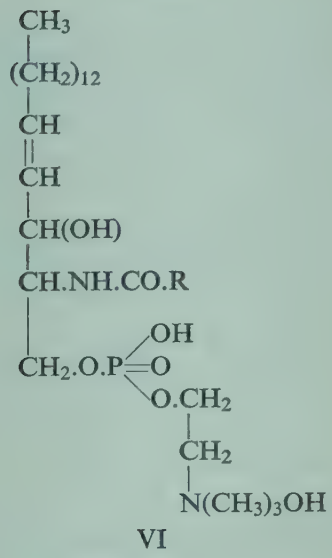
(Phosphatidyl serine)

phosphatides (V) constitute another group of phospholipids. Finally, there is sphingomyelin (VI). Carter and his associates (1947; Lovern, 1955) have recommended classing it with the other compound lipids containing sphingosine, the cerebrosides and



V

(Diphosphoinositide (?))



VI

(Sphingomyelin)

gangliosides (page 81) as sphingolipids. However, sphingomyelin behaves histochemically like the other phospholipids while the cerebrosides and gangliosides behave as 'glycolipids'.

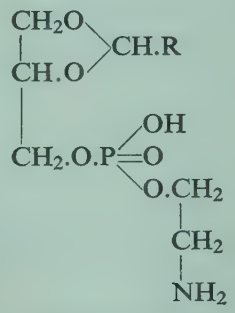
The *acid haematein* test for phospholipids was introduced by Baker (1946). It is a rigorously controlled modification of the Smith–Dietrich test (Smith & Mair, 1909; Dietrich, 1910) which depends upon postchroming, forming haematein lakes, and differentiating in a borax-ferricyanide mixture. Certain tissue constituents other than phospholipids also give positive reactions so that a control portion of tissue must be first extracted with hot pyridine. Only phospholipids, including phosphatidic acids, have been found to give a true positive result, that is a positive acid haematein reaction in the unextracted tissue and a negative reaction in the control tissue extracted with pyridine (Baker, 1946, 1947; Cain, 1947; Casselman, 1952). Some studies on the possible chemical basis of this test have been reported by Cain (1947).

A *Nile blue* method for phospholipids was devised by Menschik (1953). It depends upon the formation of stable blue compounds of Nile blue with phospholipids. Under the conditions of the test, these compounds are insoluble in hot acetone and resistant to weak acids. The specificity of this method has also been studied

(Menschik, 1953) on a variety of materials, under various conditions. The method gives positive results with phospholipids and does not require any control test to rule out false positives. It should not be overlooked, however, that this method depends upon differential extraction (*cf.* Ciaccio, 1911) so that there is always a risk that small amounts of phospholipids especially if free and not bound to some cellular structure, might be extracted with the other lipids.

The use of *copper phthallocyanine dyes* for the demonstration of phospholipids was suggested by Pearse (1955) but is no longer regarded as satisfactory (Pearse, personal communication).

5.62 Acetalphosphatides or plasmalogens. The acetalphosphatides or plasmalogens are glycerophosphatides in which two of the hydroxyl groups of the glycerol are bound by an acetal linkage to one fatty aldehyde instead of by ester linkages to two fatty

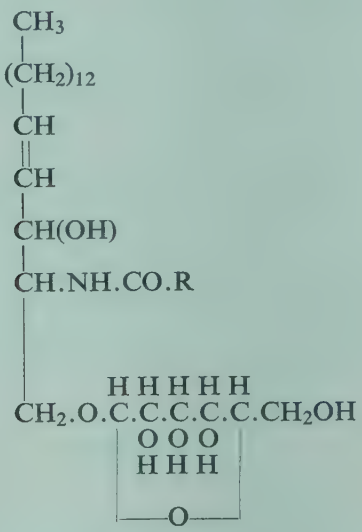


(Acetalphosphatide)

acids. Feulgen and Voit (1924), who discovered the plasmalogens, introduced the *plasmal test* for these compound lipids. It depends upon liberating the lipid aldehydes (plasmals) by hydrolysis with dilute, aqueous mercuric chloride. The aldehydes are then demonstrated with Schiff's reagent. The test is still the subject of controversies and conflicting opinions. Notable studies have been made by Cain (1949, 1949*a*), Danielli (1949, 1949*a*, 1950, 1953), Hack (1952) and Hayes (1947, 1949). Even so, much remains to be done before the plasmal test can be used confidently for the histochemical investigation of the acetalphosphatides.

5.63 Cerebrosides and gangliosides. On the basis of their histochemical properties, two types of sphingolipids, the cerebrosides

and the gangliosides, can be grouped together, possibly as 'glycolipids', although this term is not universally approved. A cerebroside contains a fatty acid, a hexose, and sphingosine:



(Cerebroside)

A ganglioside contains in addition, neuraminic acid, an amino acid with properties resembling those of carbohydrates. Although it is not possible to distinguish between these two compound lipids histochemically, their recognition as a group depends upon a positive periodic acid/Schiff reaction due to the hexose (usually D-galactose, occasionally D-glucose) and, possibly, the neuraminic acid. The other sphingolipid, sphingomyelin, contains no carbohydrate and behaves histochemically like the phospholipids, with which it has been placed (page 79).

When performing the periodic acid/Schiff test (page 44) on lipids in frozen sections of tissues fixed in formalin, the free formaldehyde should first be removed from the section by soaking it in several changes of water. Then all residual aldehydes should be blocked by dimedone or other aldehyde-blocking agent (page 48) before applying the test. A similarly treated but unoxidized control section should be used and must give a negative reaction with Schiff's reagent.

If differential extractions are carried out (page 76), the glyco-

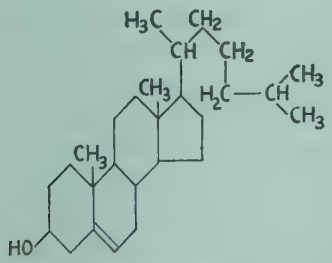
lipids can be expected to be insoluble in cold acetone but soluble in hot acetone. Thus the identification of glycolipids depends upon their sudanophilia, a positive PA/S reaction, and their being insoluble in cold acetone but soluble in hot acetone.

5.7 Derived lipids

5.71 Fatty acids. The histochemical identification of the fatty acids and their soaps is far from satisfactory. Sometimes the fatty acids are found as needle-shaped crystals. Their calcium salts usually form amorphous deposits. Fatty acids having sufficiently low melting points or in mixtures with low melting point lipids, are sudanophilic, react as acidic lipids with the Nile blue test, are extracted by cold acetone and do not give positive reactions with the PA/S test or tests for the phospholipids.

Fischler's test for the fatty acids and their insoluble soaps (Fischler, 1904) is favoured by some investigators (*e.g.* Gomori, 1952; Pearse, 1953) but not by others (*e.g.* Lison, 1953). The test depends upon the great resistance of the haematoxylin lake of cupric soaps to decolorization by Weigert's borax-ferricyanide. The method is not specific: iron and other calcium salts as well as certain tissue elements, such as muscle, also give positive reactions (Lison, 1936). Pretreatment with dilute hydrochloric acid (Mallory, 1938) or citrate buffer, pH 4·5 (Gomori, 1952) might be used to remove calcium deposits, and oxalic acid (Mallory, 1938; Lillie, 1939) to remove iron. Gomori (1952) recommends as a further control, extraction with acidified methanol-chloroform to remove all lipids so that any residual staining would represent a false positive reaction.

5.72 Sterols and their esters. The histochemical investigation of sterols and their esters has largely concerned cholesterol and its



(Cholesterol)

esters: There are two methods nominally for cholesterol: the digitonin test and an adaptation of Clark and Thompson's (1948) colorimetric method, devised by Grundland, Bulliard and Maillet (1949; Pearse, 1953), that warrants further study. There are also two methods nominally for cholesterol and its esters, based on the Liebermann and Lifschütz tests. All four methods are relatively insensitive so that although a positive result indicates the presence of the substances sought, a negative reaction does not definitely establish their absence.

5.721 Free cholesterol. Digitonin stoichiometrically forms a coordination compound with cholesterol (Windaus, 1909) and with 3-β-hydroxy sterols in general (Sobotka, 1938). For histochemical studies, Brunswick (1922) applied the reaction to tissue sections while Leulier and Revol (1930) applied it to blocks of tissue. The digitonide forms birefringent needles or rosettes that are quite coarse so that precise cytological localization is not always possible. The birefringence of cholesteryl esters, if present, can be eliminated by colouring them with one of the oil-soluble colorants (Leulier & Revol, 1930) as in Lison's (1953) modification of the digitonin test:

1. Immerse frozen sections for 2–3 hours in 0·5% digitonin in 50% ethanol.
2. Rinse in 50% ethanol, then in water.
3. 'Counterstain' with Sudan IV or oil red O.
4. Examine for birefringent crystals under the polarizing microscope.

5.722 Free and esterified cholesterol. As early as 1855, sulphuric acid colour reactions were being used for the histochemical demonstration of cholesterol (Moleschott, 1855). The two methods in use at present were introduced by Schultz in 1924 (Schultz, 1924; Schultz & Löhr, 1925). One of these is based on Liebermann's (1885) test and is frequently misnamed the Liebermann–Burchardt reaction in histochemical literature. As Cain (1950) has pointed out, in Burchardt's (1889) modification, chloroform is used as the reaction medium. The other method is based on Lifschütz's (1908) 'oxycholesterol' test. Under biochemical conditions both the Liebermann–Burchardt and the Lifschütz tests give positive reactions with all unsaturated sterols

and their esters (Sobotka, 1938). In histochemical practice, the Liebermann and Lifschütz tests can usually be regarded as being tests for cholesterol and its esters although not all cholesteryl compounds give a positive reaction (Reiner, 1952, 1952*a*) and certain other substances such as toad poisons (Cain, 1950) and carotene (Kent, 1952, 1952*a*) also give positive reactions. The usefulness of both tests, especially for precise cytological localization of cholesterol and its esters, is greatly limited by the destructiveness of the sulphuric acid.

In his adaptation of the *Liebermann* reaction, Schultz (1924) treats the tissue section with a mixture of equal parts of acetic anhydride and sulphuric acid. Romieu (1925) modified the method, treating the section with sulphuric acid followed by acetic anhydride, to give somewhat better localization:

1. Cover frozen section with 2–3 drops of concentrated sulphuric acid.
2. Within 10–15 seconds, add the same amount of acetic anhydride. Then, wash off the mixture with more acetic anhydride.
3. Apply coverslip, mounting in acetic anhydride. Ring with petrolatum if desired. Examine at once. Only a blue-green or green colour is a positive reaction.

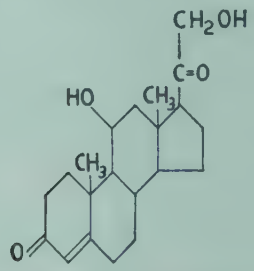
With the Liebermann and the Lifschütz (see below) tests, careful attention must be paid to the interpretation of the colours obtained. The initial red, violet or blue is not significant. If cholesterol, its esters or both are present, the colour changes to blue-green or green which alone is indicative of a positive reaction. Ultimately, the whole section turns brown, also insignificant.

In the *Lifschütz test*, preliminary exposure to air and sunlight or treatment with a ferric salt is essential. Apparently oxidation is involved, perhaps forming 7-hydroxycholesterol (Reiner, 1952*a*). The reagent for the colour reaction is a mixture of acetic and sulphuric acids. For the preliminary treatment of sections, Schultz (1924) employed either 2·5% ferric ammonium sulphate or exposure to sunlight for several days. Everett (1945) obtained similar results using 20% ferric chloride for only a few minutes. An improved Schultz–Lifschütz test, using ferric ammonium sulphate, has been devised by Weber, Phillips and Bell (1956). It is especially suitable for tissues containing relatively little cholesterol

such as bovine adrenals. To distinguish between cholesterol and its esters, Feigin (1956) has developed a method based on digitonin precipitation, differential extraction, and the Schultz–Lifschütz test.

5.8 Associated substances

5.81 Ketosteroids. From time to time, various reagents have been introduced for the demonstration of ketones, usually ketosteroids such as cortisone, in tissue sections. Subsequent investigations



(Corticosterone)

have shown that these reagents are not specific for ketones but also react with aldehydes and acetalphosphatides. Phenylhydrazine, for example, was used in studies on the adrenal cortex and considered to demonstrate ketonic lipids (Bennett, 1939, 1940). It was soon shown, however, that the reactive lipids were acetalphosphatides (Gomori, 1942, 1950; Albert & Leblond, 1946). Another reagent that was introduced as demonstrating ketosteroids is 3-hydroxy-2-naphthoic acid hydrazide (Camber, 1949; Ashbel & Seligman, 1949). Its use has led to many controversies. In reviewing the status of the reagent, Deane & Seligman (1953) emphasize that it is 'not only nonspecific for ketosteroids but is not even specific for ketones. Aldehydes . . . react even more readily with the reagent.'

Deane and her associates (Deane & Andrews, 1953; Karnovsky & Deane, 1955) have found that the carbonyl groups demonstrable in adrenal lipids following fixation of the tissue are artifacts most probably produced by autoxidation of unsaturated lipids.

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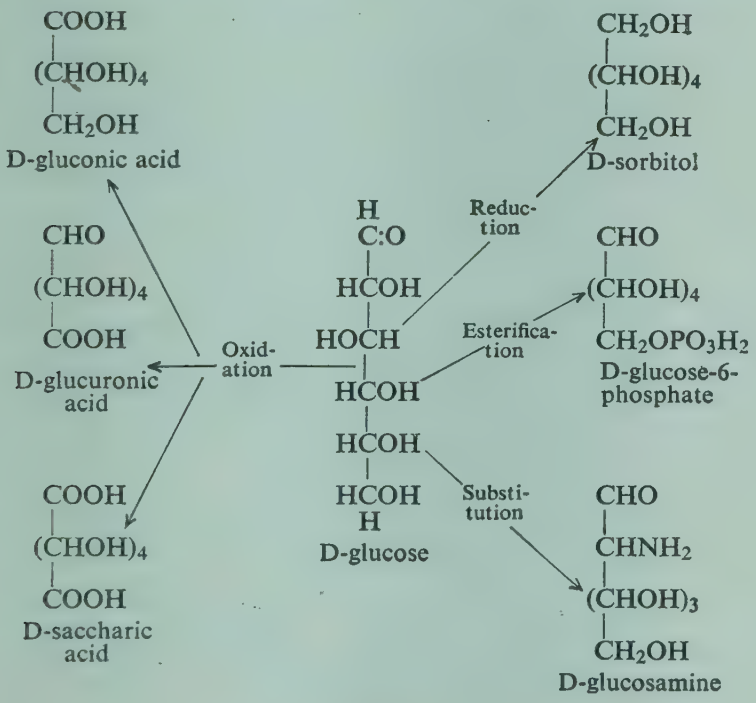
CHAPTER 6

Carbohydrates

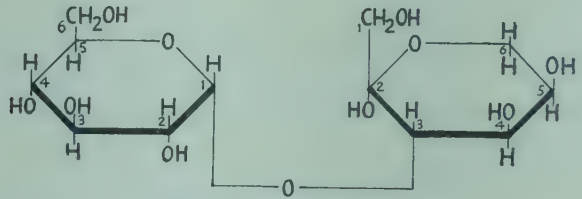
6.1 Classification

When the term carbohydrates was first used, it was thought that all compounds in this class contain carbon, hydrogen and oxygen in proportions corresponding to hydrates of carbon, $C_x(H_2O)_y$, but this excludes certain substances such as the methyl and deoxy sugars. The term is now used to include substances that have the characteristic properties of sugars or resemble them in structure and chemical behaviour. The simplest carbohydrates are the

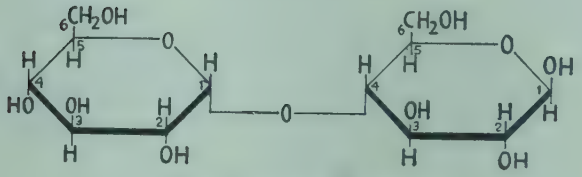


monosaccharides which are polyhydroxy aldehydes or ketones. By condensation, they form polymers that are called oligo- or polysaccharides, depending upon molecular weight. Certain esters (frequently sulphates or phosphates) and oxidation (aldonic, uronic, and saccharic acids), reduction (polyhydric alcohols and cyclitols) or substitution (e.g. amino sugars) products of the mono- and polysaccharides are also classed as carbohydrates. Examples of these products for glucose are shown in the diagram on page 91.

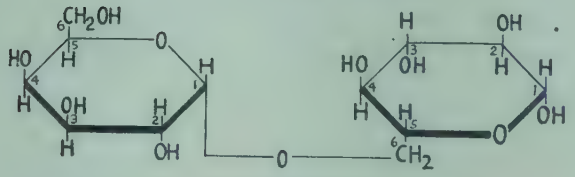
In histochemistry, many of the carbohydrates cannot be studied because of either their high solubility or their low reactivity. A classification of histochemically demonstrable carbohydrates and substances containing carbohydrates is given in Table 6.1. The *simple polysaccharides*, such as glycogen and galactogen, are polymers of the monosaccharides. Polymerization most often takes place through C_1–C_4 linkages. In some polysaccharides, however, it is through C_1–C_3 or C_1–C_6 linkages possibly resulting in different histochemical properties.



C_1–C_3 linkage in turanose



C_1–C_4 linkage in maltose



C_1–C_6 linkage in melibiose

TABLE 6.1

HISTOCHEMICALLY DEMONSTRABLE SUBSTANCES CONTAINING CARBOHYDRATES

- Simple polysaccharides
- Mucoid substances
  - Mucopolysaccharides
    - Neutral
    - Acid
      - Simple
      - Complex
  - Mucoproteins
    - Neutral
    - Acid
- Glycolipids
- Nucleic acids

Characteristically, the *mucoid substances* contain some aminosugar. The *mucopolysaccharides* occur naturally unassociated with proteins or other non-carbohydrate substances. As their name indicates, the neutral mucopolysaccharides have no free acidic groups, they usually consist of hexosamine and hexose units. The simple acid mucopolysaccharides also contain some uronic acid such as the glucuronic acid in hyaluronic acid. The complex acid mucopolysaccharides contain in addition some inorganic acid, for example, the sulphuric acid in heparin or the phosphoric acid in certain bacterial polysaccharides. In the *mucoproteins*, the carbohydrate moiety is associated with some protein. Meyer (1945) distinguishes between glycoproteins and mucoproteins on the basis of their contents of aminosugars. This is not possible histochemically. In the '*glycolipids*' (page 82) the carbohydrate is associated with a nitrogenous base and fatty acids. In the *nucleic acids* (page 110), the sugars are associated with purine and pyrimidine bases and phosphoric acid.

6.2 Preparation of tissues

Careful consideration needs to be given to the possible changes that can occur during the preparation of tissues for studies on

carbohydrates. There can be appreciable losses as well as misleading displacements of at least certain carbohydrates during fixation and subsequent processing. Unfortunately, there have been very few critical studies of the changes undergone by most carbohydrates other than glycogen. The choice of fixative and the problems associated with fixation will be discussed further in the sections devoted to the various classes of carbohydrates.

Following fixation, the tissues can be embedded in celloidin or paraffin. Frozen sections are usually unsuitable. If there is any concern over possible loss of glycogen or other polysaccharides during the test, the section can be covered with collodion:

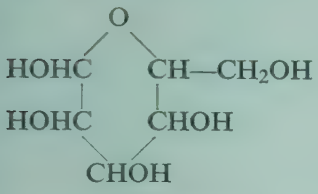
1. Take paraffin section mounted on a slide through xylene or other solvent to absolute ethanol.
2. Transfer to 1% collodion in ether and ethanol for 5–10 minutes.
3. Drain slide for 1 minute and transfer to 80% ethanol for 5 minutes to harden the collodion.
4. Wash slide in water.

A collodion film should not be applied before enzymic hydrolysis because it is often impermeable to enzymes such as diastase. The collodion film is soluble in pyridine. Consequently it should not be applied to a section before esterification (page 49) if pyridine is to be used as the reaction medium.

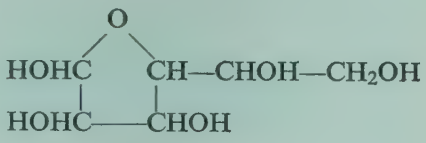
6.3 General methods

6.31 Selective oxidant/Schiff Tests. Methods depending upon the detection of aldehydes are important in the histochemical investigation of carbohydrates. The carbohydrates remaining in sections of fixed tissues, however, do not give direct aldehyde reactions because there are no free aldehyde groups, the aldoses being present in their pyranose or furanose forms so that their potential aldehyde groups are unreactive. Instead, aldehyde groups must be formed by selective oxidation from some of the hydroxyl groups present in the carbohydrates. For this purpose, periodic acid is most often used. Like the oil-soluble colorant test for lipids (page 68), the periodic acid/Schiff test is usually regarded as the generic test for carbohydrates even though not all carbo-

hydrates give a positive reaction and not all positive reactions are due to carbohydrates.



(Pyranose)



(Furanose)

Oxidation by periodic acid and the basis of the PA/S test have been discussed in Chapter 4 (page 40). In tissues, the necessary α-glycol or other groups could be provided by a wide variety of carbohydrates including the simple sugars and their phosphorylated derivatives, the polysaccharides and mucopolysaccharides, and the glyco- and mucoproteins. Ordinarily, however, the low molecular-weight sugars and their derivatives are lost during fixation but the polysaccharides of higher molecular weight and other less soluble constituents remain. This relatively broad selectivity of the PA/S test can be narrowed by supplementary enzymic hydrolysis (page 97).

In the case of C_4-linked polysaccharides, oxidation by periodic acid is not accompanied by depolymerization because the oxidizing agent acts on C_2 and C_3 leaving the C_1–C_4 linkages intact. These polysaccharides present an α-glycol group for each unit, regardless of chain length. With C_3-linked polysaccharides, only the terminal units can present α-glycols for oxidation by periodic acid so that the relative amount of aldehyde available for reacting with Schiff's reagent varies inversely with the chain length. There are some polysaccharides, such as cellobiose and certain methylglycosides, that are very resistant to periodic acid (Jeanloz, 1950) even though they satisfy the structural requirements for oxidation. Other carbohydrates are not oxidized by periodic acid because the required vicinal hydroxyl groups are either involved in polymeric linkages, as in ribonucleic acids and C_3-linked polymers like agar, or lacking altogether as in deoxyribofuranose.

The PA/S test is described on page 44. A modification of it for studying water soluble polysaccharides has been developed by Mowry and his associates (1952, Mowry, Longley & Millican, 1952; Mowry & Millican, 1953; see page 101).

Various other selective oxidants (page 39) can be used in studies on carbohydrates but chromic acid and periodic acid have been used most extensively. The results obtained with chromic acid, periodic acid, and potassium permanganate as oxidizing agents have been compared by Lillie (1951). The data presented in Table 6.2, suggest that chromic acid is somewhat more selective than periodic acid and might be preferable as the oxidizing agent

TABLE 6.2

COMPARISON OF REACTIONS OF TISSUE CARBOHYDRATES WITH BAUER AND PERIODIC ACID/SCHIFF TESTS

Carbohydrate	*Bauer*	*PA/S*
Glycogen	+	+
Galactogen	+	+
Starch	+	+
Cellulose	+	+
Chitin	+	+
Tunicin	+	+
Epithelial mucins	+	+
Thyroid colloid	+	+
Connective tissue glycoproteins	0-tr.	+
Bacterial polysaccharides	0	+
Fungal polysaccharides	0	+
Cerebrosides (Gaucher's disease)	0	+

(For additional comparisons, see Lillie (1951))

for studies on glycogen or epithelial mucins to the exclusion of the glycoproteins of connective tissues. Generally, on the same material, the intensity of the Schiff reaction is greater after oxidation with periodic acid than after chromic acid so that the Bauer test tends to be less sensitive than the PA/S test. Periodic acid is the reagent of choice for studies on the polysaccharides and glycoproteins of connective tissues and various micro-organisms. Regardless of the oxidizing agent, some substances such as mast cell granules, cartilage ground substance and amyloid give either negative or faintly and irregularly positive reactions. Bauer's test, using chromic acid, is described on page 47.

Lead tetra-acetate and sodium bismuthate are also effective as selective oxidizing agents and their use in histochemistry needs

to be explored further. The selectivity of lead tetra-acetate, in so far as carbohydrates are concerned, can be varied by the conditions under which the reagent is used (Casselman, 1954).

6.4 Differential methods

Quite often the distribution and morphology of tissue carbohydrates are of considerable help in their recognition; for example, glycogen in hepatic cells, starch in plant cells and mucins in intestinal or tracheal epthelium. Other carbohydrates and other cells, however, often require selective tests, either enzymic hydrolysis or staining methods.

6.41 Enzymic hydrolysis. One of the oldest enzymic methods in histochemistry uses saliva as a source of amylase, an enzyme that hydrolyses starch and glycogen but practically no other polysaccharide. An important disadvantage is the highly variable activity of saliva. Preparations of amylase from other sources permit more consistent results in distinguishing starch and glycogen from other carbohydrates in tissue sections (page 100).

Various enzymes in addition to amylase, such as the hyaluronidases (e.g. Bunting, 1950; Gersh & Catchpole, 1949; Grishman, 1948, 1952) and pectinases (McManus & Saunders, 1950; McManus & Cason, 1951; Eidinger & Ghosh, 1956) have been suggested for the selective removal of certain polysaccharides and mucopolysaccharides. The hyaluronidases appear to be specific in their action on hyaluronic acid and chondroitin. The effects of these enzymes in abolishing some or all of the histochemical reactions of these substances depend upon the source of the enzymes and the duration of treatment (Matthews, Roseman & Dorfman, 1951). The actions of pectinase and other enzymes have yet to be established.

The general use of enzymes as differential reagents is discussed in Chapter 4 (page 58). Their use in the study of carbohydrates presumes that the disappearance of a histochemically characteristic reaction following treatment of a tissue section with a preparation having established carbohydrate-splitting activity indicates that the specific subtrate of that enzyme had been present. Such a conclusion might be justifiable provided that pure enzymes with completely known activities were available. However, relatively crude and only partially purified preparations are often used in histochemical studies and can bring about

changes in tissue constituents other than by specific hydrolysis (Benditt & French, 1953). Consequently, the results must be interpreted very cautiously.

6.42 Selective staining. Most of the selective staining methods that are still used in the histochemical investigation of carbohydrates are purely empirical tests such as Best's carmine stain for glycogen (page 100), Mayer's mucihaematein and mucicarmine methods for mucins (page 105) and, to some extent, the Alcian blue test and Hale's test for acid mucopolysaccharides (page 104). These methods are discussed under the appropriate classes of carbohydrates. Metachromatic staining, also used in studies on the acid mucopolysaccharides, is discussed in Chapter 4 (page 53).

6.5 Simple Polysaccharides

In general, the histochemical recognition of simple polysaccharides depends upon:

(*a*) a positive PA/S reaction,
(*b*) negative reactions with tests for mucoid substances and glycolipids,
(*c*) hydrolysis by or resistance to certain carbohydrate-splitting enzymes, and
(*d*) selective tests for some of the individual polysaccharides.

6.51 Glycogen. Glycogen is practically the only simple polysaccharide normally occurring in vertebrate tissues. It appears to occur in at least two forms. As early as 1833, Ehrlich suggested that some of it is bound to another substance in cells. Willstatter and Rohdewald (1934) described a 'lyoglycogen', which is readily soluble in warm water or trichloracetic acid, and a 'desmoglycogen', which is insoluble in these reagents and is believed to be bound to protein. On the other hand, Meyer and Jeanloz (1943) have shown that glycogen is not necessarily chemically combined with protein but could be merely enmeshed in denatured protein under some conditions. Meyer (1943) regards desmoglycogen as being glycogen of high molecular weight rather than protein-bound.

6.511 Fixation. There have been a number of studies on the fixation of glycogen but there is little unanimity of opinion

regarding the most satisfactory mixture (Bensley, 1934; Deane, Nesbett & Hastings, 1946; Lillie, 1947; Vallence-Owen, 1948; Langeron, 1949; Lison & Vokaer, 1949; Gomori, 1952). Ethanol alone or in mixtures has been repeatedly recommended partly on the basis of histochemical studies but largely because, in the free state, glycogen is soluble in aqueous mixtures and insoluble in alcoholic ones. In tissues, however, glycogen is usually associated with the protein constituents of cells. Most fixing agents that are good protein precipitants also preserve glycogen well, perhaps because the glycogen cannot diffuse through the precipitated protein. Pasteels and Leonard (1935) found that Bouin's fluid gives good results. Lison (1953) has recommended ice-cold Gendre's fluid, essentially an alcoholic Bouin's fluid. Lillie (1947) found Bouin's fluid, as well as mixtures containing mercuric chloride, unsuitable. Gomori (1952) agrees with the French authors regarding the suitability of mixtures containing picric acid and with Lillie regarding the unsatisfactory fixation of glycogen by mixtures containing mercuric chloride. Ice-cold Rossman's fluid, recommended by Deane, Nesbett and Hastings (1946), gives excellent results.

The size and distribution of the particles of glycogen are influenced by the nature of the fixative mixture. When tissues are fixed in mixtures containing acid, alcohol or both, the glycogen is usually preserved as coarse particles. When mixtures containing formaldehyde are used, the glycogen granules are usually finer and more uniform in size and distribution, resembling the glycogen in frozen-dried tissues.

To avoid loss of glycogen, it is advisable to use ice-cold fixatives and to keep the tissues in the refrigerator during fixation. This is especially important for liver in which glycogenolysis otherwise occurs rapidly and can result in appreciable loss of demonstrable glycogen, especially near the centre of the block of tissue. This loss is more noticeable with Bauer's or Best's method for glycogen than with the PA/S test. In other tissues, loss of glycogen occurs less rapidly.

Postmortem loss is not the only difficulty encountered in studies on glycogen. Displacement of the polysaccharide to one side of the cell occurs with some fixatives, especially ethanol at room temperature. This artifact, which has no biological significance, has been referred to variously as 'Alkoholflucht', 'polarization'

and 'streaming'. Takahashi and Iwase (1955) have suggested that it is related to the nature of the tissue, occurring most noticeably in solid, parenchymatous tissues, such as the liver, which permit the fixative to diffuse into a given cell from only one direction. Displacement of glycogen can be diminished by using cold mixtures such as recommended above. Fixation occurs more slowly in the cold, however, so that the tissues must be left in the fixative longer than at room temperature. The artifact does not occur in tissues prepared by freeze-drying or by freeze-substitution. The latter method (Simpson, 1941, 1941*a*) deserves further investigation and more widespread use in histochemistry.

6.512 PA/S test and amylase hydrolysis. Glycogen gives a positive PA/S reaction (page 44) and is readily removed by amylase. This enzyme (Bernfeld, 1951) hydrolyses the glycogen to maltose which is dissolved out of the section. Any amylase-resistant material remaining in the section is not glycogen or, in the case of plant material, starch. Good results can be obtained using Lillie and Greco's (1947) method. The section must not be coated with collodion because the enzyme cannot diffuse through the film. The section should be incubated in a one percent solution of the enzyme in buffered neutral saline (0·8% NaCl; 0·08% $NaH_2PO_4.H_2O$; 0·13% Na_2HPO_4) at 37° C. for one hour or longer. A control section of the same tissue should be similarly incubated in the buffered saline without enzyme. Another control section of a tissue similarly fixed and prepared and known to contain glycogen should be incubated in the buffered enzyme solution to check the activity of the enzyme.

6.513 Best's carmine method. Best's carmine method is an empirical but remarkably selective staining method for glycogen (Best, 1906; Bensley, 1939; Mallory, 1938). Mucin, fibrin and mast cell granules are also stained but so much less intensely that they are not apt to be confused with glycogen. For this test paraffin sections coated with collodion should be used.

1. Prepare the stock solution of carmine by adding 2·0 gms. carmine, 1·0 gm. potassium carbonate and 5·0 gms. potassium chloride to 60 ml. distilled water. Boil the mixture gently for 3–5 minutes. Cool and add 20 ml. concentrated ammonium hydroxide. Stopper tightly and store in the refrigerator. This solution can be kept for 2–3 months.

2. Immediately before use, prepare the staining solution by mixing 10 ml. stock solution, 15 ml. concentrated ammonium hydroxide and 15 ml. 95% ethanol. Filter if necessary.
3. Bring the section to water and slightly overstain the nuclei with an acid alum haematoxylin (*e.g.* Ehrlich's).
4. Rinse in tap water.
5. Stain for 10–15 minutes in staining solution.
6. Rinse in 3 changes of methanol.
7. Dehydrate and remove collodion in 3 changes of acetone.
8. Clear and mount in synthetic resin.

Glycogen deposits are stained red; nuclei, blue.

6.52 Galactogen. This polysaccharide consists entirely of galactose units. Its histochemical properties have been studied by Grainger and Shillitoe (1952). Like glycogen, galactogen gives a positive PA/S reaction. The two polysaccharides can be differentiated by a series of tests outlined by these authors. Some mucopolysaccharides or glycoproteins might react in the same way as protein-bound galactogen. Commercial pectinase preparations, but neither malt diastase nor saliva, remove galactogen from tissue sections.

6.53 Starch. Like glycogen, starch gives a positive PA/S reaction and is hydrolysed by saliva and the amylases. Enzymic hydrolysis of whole starch grains is slow. However, the polysaccharide is hydrolysed rapidly after it has been liberated from the plastids. The shape of the starch grains can be of considerable help in identifying their source. Owing to the molecular orientation, starch grains are birefringent.

For well over a century, iodine has been used as a stain for starch. It can be used as a vapour or as a solution in paraffin oil (Mancini, 1948) or aqueous potassium iodide (0·2% iodine in 2% aqueous potassium iodide). Iodine stains native starch blue. Glick (1949) recommends Milovidov's (1928) method for permanent preparations.

6.54 Dextrans. The ordinary PA/S tests were used in early studies on the distribution of intravenously administered dextrans in tissues (e.g. Friberg, Graf & Aberg, 1951; Persson, 1952). These polysaccharides, however, are readily soluble in water. Therefore,

Mowry and his co-workers (Mowry, 1952; Mowry, Longley & Millican, 1952; Mowry & Millican, 1953) developed a non-aqueous technique that they have used successfully for studies on dextran.

6.6 Mucoid substances

The mucoid substances do not always behave histochemically as might be expected. For example, chondroitin sulphuric acid, a complex acid mucopolysaccharide, fails to give a positive PA/S reaction because some of the glycol groups are substituted (Meyer & Odier, 1946).

At present, it is not always possible precisely to distinguish between each of the chemically defined classes of mucoid substances as listed in Table 6.1. On the other hand, and perhaps in keeping with the histological as well as chemical interests of histochemistry, it is sometimes possible to distinguish between mucoid substances of epithelial and mesenchymal origins (Grishman, 1948, 1952), although the behaviour of these substances can vary appreciably in closely related tissues (Lillie & Mowry, 1949; Lillie, 1951; Gomori, 1954).

6.61 Fixation. For mucoid substances, some authors believe that the choice of fixative is not critical. Others believe that the mucopolysaccharides and mucoproteins are best preserved by conventional alcoholic and strongly acidic fixatives. 4% basic lead acetate (= lead subacetate), with (Sylvén, 1941) or without (Holmgren & Wilander, 1937) formalin, has been recommended for fixation of mucopolysaccharides, especially for studies with metachromatic stains. Formaldehyde is necessary for the fixation of mucoproteins such as occur in the anterior pituitary (Pearse, 1953).

Hale reported that treatment of some mucoid substances with sodium hydroxide before oxidation by periodic acid increases the intensity of their PA/S reactions (Hale, 1953). He first suggested this effect might be related to chain lengths, amounts of hexosamines or the degree of conjugation with proteins (Hale, 1953*a*) and later showed that it is at least partly related to formaldehyde fixation (Hale, 1955). The exact mechanism of this important effect has yet to be established.

6.62 Identification. The *acid mucopolysaccharides*, whether simple

or complex, generally stain metachromatically with oxazine and thiazine dyes, and are stained by Alcian blue. They react positively with Hale's test. The mucicarmine and mucihaematein tests are empirical staining methods that were introduced by Mayer (1896) and later modified by Southgate (1927) and Laskey (1950). These tests are remarkably selective for mucoid substances which also stain metachromatically, especially if the tissues are fixed in ethanol or formaldehyde-ethanol and not stained excessively.

Hyaluronidase readily hydrolyses hyaluronic acid, a simple acid mucopolysaccharide, but less readily the complex acid mucopolysaccharide, chondroitin sulphuric acid type A. The differential properties of streptococcal and testicular hyaluronidases (Meyer, Chaffee, Hobby & Dawson, 1941), so useful in biochemical studies, do not seem to be so readily realized histochemically (Grishman, 1952).

The *neutral mucopolysaccharides* do not stain metachromatically, are not appreciably stained by Alcian blue, and do not give a positive reaction with Hale's test. Unlike the simple polysaccharide, glycogen, they are not hydrolysed by amylase.

The presumptive identification of a *mucoprotein* depends largely upon positive protein reactions (Chapter 8) at the same site as positive carbohydrate reactions. However, some supposed mucoproteins do not give positive reactions with at least some protein tests. Moreover, there is always the possibility that proteins and carbohydrates exist independently at the same site and are not there as mucoproteins.

6.63 Hale's test. Hale's (1946) dialysed iron method apparently depends upon the adsorption of colloidal iron oxide by acidic tissue constituents and the subsequent demonstration of the iron by the Prussian blue reaction (page 158). Although the test was suggested as a method for acid polysaccharides, it is not specific for them and is not even selective for any chemically definable group of substances. The method demonstrates exceptionally well some but not all (Grishman, 1948) forms of metachromatic mucin. In most tissue sections, there is some diffuse, light blue background staining. The results obtained using Hale's test closely resemble those obtained with the mannitol-ferric chloride method of Lillie and Mowry (1949) and the iron method of Wigglesworth (1952).

In comparative histochemical and biochemical studies of human costal cartilages Joel, Masters and Shetlar (1956) found remarkably good agreement between the results obtained histochemically with the PA/S and Hale's tests and the results of biochemical determinations of glycogen, acid polysaccharides and polysaccharides containing neutral hexosamines.

For Hale's test, tissues should be fixed in Carnoy's fluid or in an alcoholic mixture and embedded in paraffin.

1. Bring section to water and immerse in a mixture of equal parts of 2N. acetic acid and pharmaceutical grade dialysed iron (5% Fe_2O_3), for 10 minutes.
2. Wash section well in distilled water and immerse in a mixture of 85 ml. 1% potassium ferrocyanide and 15 ml. 1N. hydrochloric acid, for 10 minutes.
3. Wash section in distilled water and counterstain with safranin. Dehydrate, clear and mount in synthetic resin.

Positively reacting substances are coloured blue.

Rinehart and Abu'l-Haj (1951) modified Hale's method by specially preparing the colloidal iron reagent and using a Van Gieson counterstain. Longley (Lillie, 1954) has further modified this by using the Feulgen nucleal test (page 118) as a nuclear 'stain'. Ritter and Oleson (1950) combined Hale's test with the PA/S test.

6.64 Alcian blue methods. Steedman (1950) introduced Alcian blue 8GS, a phthallocyanine dye, as a selective stain for mucins. Vialli (1951) found it selective for ephithelial mucins and certain polysaccharides. According to Steedman (1950) the tissues should be fixed in Bouin's or Zenker's fluid or 'susa', but not in formalin. They should be embedded in paraffin.

1. Bring section to water and stain for 10–40 *seconds* in 1% aqueous Alcian blue 8GS, saturated with thymol. The reagent can be stored for 6–8 weeks but should be filtered at least every 7–10 days.
2. Rinse in distilled water and immerse for 2 hours in 0·5% borax in 80% ethanol (see below).
3. Counterstain nuclei and cytoplasm as desired. Dehydrate, clear and mount in synthetic resin or Canada balsam.

Alcian blue initially stains the mucins a clear greenish blue. The dye is converted to an insoluble blue pigment, Monastral fast blue, by the alkaline ethanol. Unlike the Alcian blue, this pigment is resistant to the later histological reagents.

Lison (1954) modified the original Alcian blue method to give greater selectivity in the demonstration of connective tissue elements. He compared the results with those for metachromatic staining and the PA/S test. Like Vialli (1951), Lison found that there is close agreement between staining with Alcian blue and staining with metachromatic dyes. He found examples of all four possibilities when comparing the PA/S reaction with the Alcian blue staining of various tissue constituents: strongly PA/S-positive and weakly Alcian blue-positive, weak PA/S and strong Alcian blue, strong with both tests, or weak or negative reactions with both.

In their remarkable studies on the distribution of sulphated mucopolysaccharides in the mouse, Curran and Kennedy (1956) compared ^{35}S autoradiographs with sections stained with Alcian blue (Steedman, 1950), or a metachromatic dye or by Hale's test or the PA/S test. Of the four methods, Alcian blue gave closest correlation with the autoradiographs suggesting that it selectively stains most sulphated mucopolysaccharides.

6.65 Mucihaematein test. Laskey (1950) modified Mayer's mucihaematein staining method by substituting haematoxylin for haematein and omitting the nitric acid. Her simple technique gives consistent results, staining mucin a deep violet and nuclei a greyish blue. The stain retains its selectivity for many months instead of only a few days as does Mayer's.

1. Prepare the reagent by dissolving 1 gm. haematoxylin in 100 ml. 70% ethanol, adding 0·5 gm. aluminium chloride and 5 ml. 1% aqueous sodium iodate, and finally making the mixture up to 500 ml. with 70% ethanol.
2. Bring section to water and rinse thoroughly with distilled water. Cover the section with 2 ml. of the staining solution.
3. In 5–10 minutes, pour off the stain and wash for 5 minutes with each of 3 changes of distilled water.
4. Dehydrate, clear and mount in synthetic resin.

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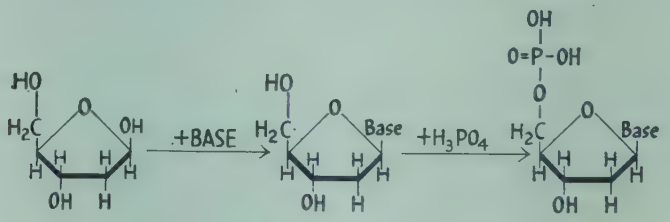
CHAPTER 7

Nucleic Acids

A great deal of current interest in the nucleic acids arises from their intimate association with genes and chromosomes and from their apparent involvement in protein synthesis. Histochemical techniques have played an important part in establishing these relationships. There are only two types of nucleic acids to be differentiated histochemically from each other and from other constituents of cells and tissues. These are the deoxyribonucleic acids (DNA) and the pentose- or ribonucleic acids (RNA).

7.11 Biochemistry. The nucleic acids are polynucleotides. Each nucleotide is a phosphoric ester of a nucleoside which is the condensation product of a purine or pyrimidine base with a pentose or deoxypentose. The pyrimidine bases include uracil, thymine, cytosine and methylcytosine. The purine bases include hypoxanthine, xanthine, guanine and adenine. As their names indicate,

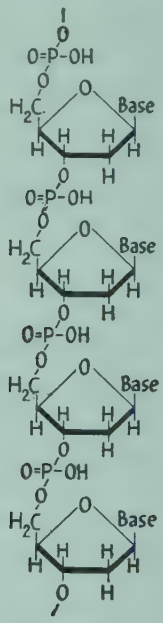


Deoxypentose Nucleoside Nucleotide

it is the sugar that differs in the two types of nucleic acids, being deoxyribose in DNA and some pentose in RNA.

The *deoxyribonucleic* acids occur in cell nuclei, associated with histone, a basic protein, and possibly with other nuclear proteins. These nucleic acids exist as straight-chained polynucleotides of high molecular weight, probably containing several thousand residues united mainly by C_3'–C_5' diester, orthophosphoric acid

linkages. The molar proportions of the purine and pyrimidine bases vary with the source but the DNAs tend to fall into one

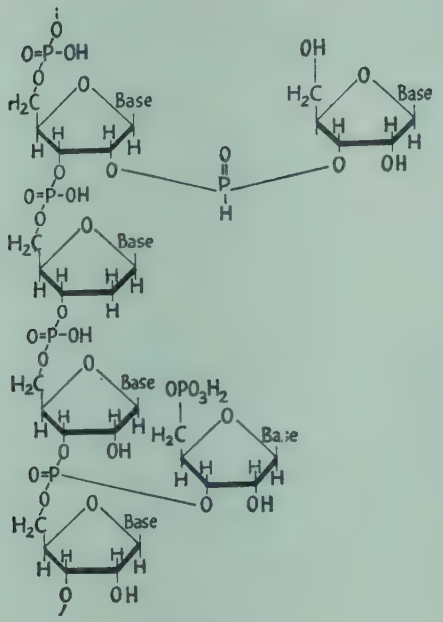


(DNA)

of two main groups in which either adenine and thymine or guanine and cytosine predominate (Chargaff, 1950, 1951).

The *ribonucleic* acids occur naturally as ribonucleoproteins in nucleoli and in the basiphilic cytoplasmic granules. Little is known about the nature of the protein. In the granules, the ribonucleoprotein might be associated with compound lipids (Davidson, 1953). The RNAs exist as linear, and possibly branched-chain, polynucleotides having lower molecular weights that the DNAs and probably containing not over 100 residues. The internucleotide linkages are phospho-diester groups between C_3' and C_5' (Brown & Todd, 1953). The molecular proportions of the purine and pyrimidine bases vary widely in RNAs from different sources.

7.12 Histochemistry. It might be expected that each of the three components of the nucleic acids could provide the basis for a histochemical test for their identification. In practice, the purine



(RNA)

and pyrimidine bases have not provided satisfactory histochemical methods, although they can be localized by ultraviolet microscopy. The most reliable method for DNA, the nucleal test (page 113), depends upon the carbohydrate moiety. Turchini and his associates have made extensive use of 9-phenyl-2,3,7-trihydroxy-6-fluorone which condenses with both ribose and deoxyribose as well as hexoses. Histochemical methods for the nucleic acids depending upon the specific identification of phosphoric acid have been described but are unreliable. The most generally used method (page 118) for RNA depends upon the high affinity of the nucleic acids for certain basic dyes. Since there are other basiphilic constituents of tissues, the identification of RNA using such a staining method depends upon the selective removal of the nucleic acid by ribonuclease or by chemical extraction (page 120).

7.2 Preparation of tissues

Formaldehyde, organic solvents, and most acidic fixatives do not cause appreciable losses of the nucleic acids. With acetic acid-

ethanol mixtures, significant redistribution can sometimes occur (Mellors, 1950; Mellors, Berger & Streim,1950; Chayen & Norris, 1953). Less serious but misleading are the morphological artifacts that can develop during postmortem shrinkage and fixation (Doyle & Metz, 1935; Ris & Mirsky, 1949, 1949*a*; Kurnick, 1950; Kurnick & Herskowitz, 1952). With certain fixatives, such as Rossman's fluid, there can be some depolymerization of DNA (Kurnick, 1952). Kurnick (1955) concludes that although there is no perfect fixative, 10% formalin brought to pH 7·0 is satisfactory for most purposes. Following such fixation, tissues can be dehydrated, cleared and embedded in paraffin in the usual way.

7.3 Deoxyribonucleic acids: the nucleal test

The nucleal test is the most reliable and specific histochemical method for DNA. It was introduced by Feulgen and Rossenbeck in 1924. It was soon widely used by cytologists for studies on nuclei so that nearly 500 papers describing applications of the nucleal test were published within little more than a decade (Milovidov, 1938). The test is commonly referred to as the 'Feulgen test'. The original authors, however, recommended the name 'nucleal', recognizing that the test depends upon the formation of some aldehyde from DNA. This name avoids any confusion with the other 'Feulgen test', the plasmal test of Feulgen and Voit (page 81).

7.31 Mechanism of test. In spite of numerous studies, many details of the mechanism of the nucleal test for DNA remain rather obscure (Lessler, 1953; Kurnick, 1955; Swift, 1955). The test appears to depend upon freeing some of the deoxyribose aldehyde groups by mild acid hydrolysis and then demonstrating these newly formed groups with Schiff's reagent (Feulgen & Rossenbeck, 1924; Li & Stacey, 1949; Overend & Stacey, 1949; Overend, 1950; Lessler, 1951).

Mild acid hydrolysis of DNA can selectively remove the purine bases yielding a rather ill-defined degradation product named thymic acid (Kossel & Neumann, 1893, 1896; Feulgen, 1917). In studies on tissue sections, Di Stefano (1948, 1948*a*) was able to demonstrate 'a progressive loss of the bases from the nucleus and no other change' up to the optimum time for hydrolysis in the

nucleal test. One half of the bases disappear, presumably the purines. With prolonged hydrolysis there is also loss of thymic acid and basic protein from the nucleus.

While breaking purine-deoxyribose bonds is generally considered to be an important effect of the hydrolysis leading to the production of aldehyde groups, Overend and Stacey (1949) have pointed out that the actual removal of purines is not essential for the nucleal test and that some degree of depolymerization might have the same result. They have suggested that 'under acid conditions, those sugar linkages engaged in polymeric bonding first become broken . . . followed by the rupture of the linkages attached glycosidically to purine bases. The 2-deoxyribose components thus revealed are still attached through phosphate linkages at C_3 and C_5 in the main chain of the nucleic acid and consequently are firmly held in the furanose form, which immediately is transformed into a significant proportion of the aldehydo form. The Schiff's reagent then reacts with the revealed aldehyde groups . . .'

7.32 Intensity of reaction. There are two important technical factors influencing the intensity of the nucleal reaction: the type of fixation and the duration of hydrolysis. As the duration of hydrolysis is increased, the intensity of the reaction increases to a maximum. The time at which this maximum is attained, and whether it is broad or sharp, depend upon the fixation (Bauer, 1932; Hillary, 1939; Di Stefano, 1948, 1948*a*). Sharply defined (± 1 minute) optimum times of hydrolysis are obtained for tissues fixed in acetic-ethanol (Carnoy's), acetic-sublimate, or trichloracetic-sublimate using 1N. hydrochloric acid at 60° C. (A, Fig. 7.1). Under similar conditions there are very broad (10–20 minutes) optima for tissues fixed in mixtures containing chromic acid such as Allen's, Flemming's, Champy's and Sanfelice's fluids (B, Fig. 7.1). The hydrolysis proceeds more rapidly, of course, at higher temperatures. Feulgen and Rossenbeck (1924) recommended 60° C. and this has been generally used ever since.

The optimum time of hydrolysis and the maximum intensity of the nucleal reaction vary to some extent with the species. For example, the nuclei of *Spirogyra* require longer hydrolysis than those of most plant cells (Hillary, 1939) while the nuclei of sea urchin eggs require less time than those of most animal cells

(McMaster: quoted by Swift, 1955). While some studies, using visual estimates, have suggested that the nuclei of different tissues vary in their ease of hydrolysis, thymus being most resistant (De Lamateer, Mescon & Barger, 1950), Swift (1955) has found no

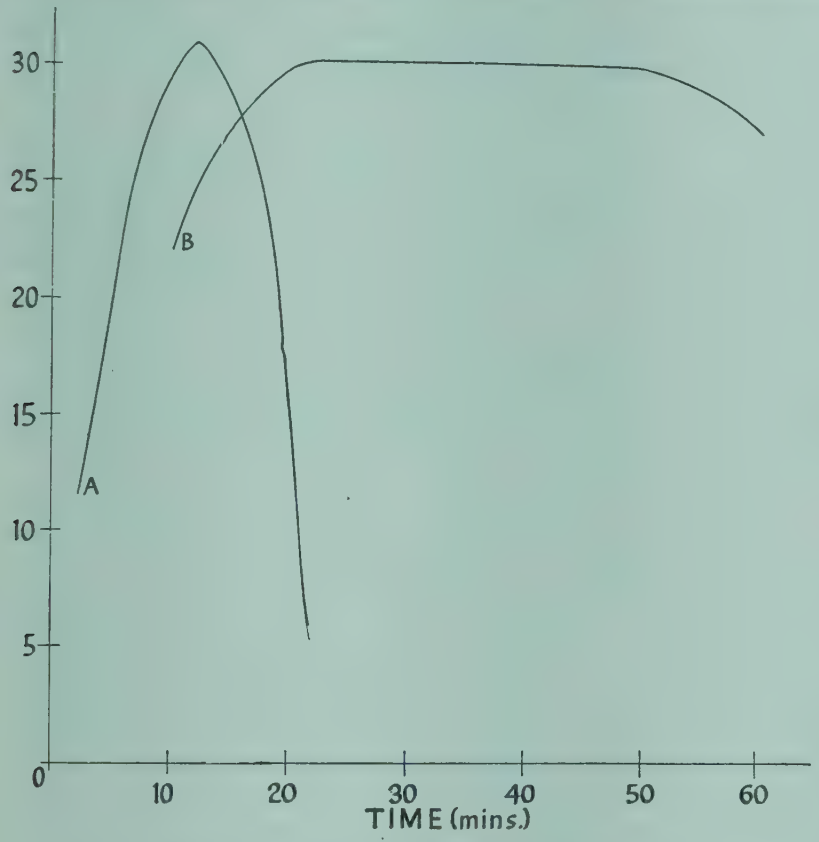


FIG. 7.1. Relation of Intensity of Nucleal Reaction to Time of Hydrolysis (after Di Stefano, 1948, 1948*a*)

microspectrophotometric evidence of differences between tissues, except sperm nuclei.

The nature of the fixative also influences the maximum intensity of the reaction which can be twice as great after fixation in formaldehyde as after acetic-ethanol (Swift, 1950). The nucleal reaction is more intense after fixation in 20% formalin than

after 50% formalin (Naora, Matsuda & Sibatani: quoted by Sibatani & Fukuda, 1953).

Lhotka and Davenport (1951) found that with the nucleal test modified so that it could be applied to blocks of tissue, the reaction is less intense if the tissues are fixed in picrosulphosalicylic acid immediately rather than after 5 minutes delay, suggesting that early postmortem changes possibly influence the reactivity of the DNA in nuclei.

Another cause of variation in intensity of the nucleal reaction is related to the penetration of the fixative into the block of tissue. Swift (1953, 1955) has found that with either neutral formalin or acetic-ethanol, the reactions given by nuclei near the periphery of a block are as much as 30 per cent greater than those given by nuclei near the centre, as measured spectrophotometrically. There is no such variation in intensity in frozen-dried or fresh-frozen tissue preparations. The ease with which a fixative can penetrate tissues becomes an important factor when comparative studies are to be made as, for example, between readily penetrable testicular tissue and relatively impenetrable hepatic tissue.

7.33 Specificity. The nucleal test has been widely used as a specific test for DNA, a positive reaction being considered indicative of the presence of the nucleic acid and a negative reaction, of its absence, provided the test has been carried out properly with the necessary controls. Moreover, quantitative significance has often been assumed for 'strong' and 'weak' reactions. While the majority of investigators have accepted the nucleal test as being reliable for the intracellular localization of DNA, a few have consistently raised doubts regarding the validity of the method. The resultant controversy has been reviewed by Gomori (1952), Lessler (1953), Pearse (1953), Kurnick (1955) and Swift (1955). The most recent evidence that the nucleal test is specific for DNA and that its mechanism is not a simple staining reaction has been published by Kasten (1956). In the words of Overend and Stacey (1949), the nucleal reaction 'does locate the precise site of deoxyribonucleic acid'.

7.34 Procedure. Although various acids such as citric (Widström, 1928), perchloric (Di Stefano, 1948*a*, 1952), phosphoric (Hashim, 1952), chromic, nitric and sulphuric acids as well as bromine (Barka, 1956) can be used, hydrolysis for the nucleal test is still

TABLE 7.1

OPTIMUM TIMES FOR HYDROLYSIS* IN THE NUCLEAL TEST

Fixative	*Bauer (1932)*	*Other Authors*
Acetic-sublimate	5	
Allen		20 (Lison, 1953)
Apathy	5	
Bouin		12 (Lison, 1953)
Bouin–Allen	22	
Bouin–Allen sublimate	14	
Bouin-Duboscq	6	
Carnoy (3 : 1)	6	12 (Di Stefano, 1948, 1948*a*)
Carnoy (6 : 3 : 1)	8	
Carnoy-Lebrun	6	
Champy	25	
Chrome-acetic	14	
Ethanol, absolute		5 (Lison, 1953)
Ethanol : formol : acetic		7 (Lison, 1953)
(85 : 10 : 5)	7	30 (De Smul & Lecompte, 1953)
Flemming	16	20–50 (Di Stefano, 1948, 1948*a*)
Flemming–Heitz	25	
Formaldehyde		8 (Pearse, 1953)
Helly	8	
Hermann		
Kahle	5	
Petrunkevitch	3	
Picric acid		
Regaud	14	
Regaud-sublimate	8	
Sanfelice	6	
Susa		18 (Pearse, 1953)
Zenker	5	
Zenker-formol	5	

* 1N. hydrochloric acid at 60° C., times in minutes.

most often carried out with 1N. hydrochloric acid at 60° C. as originally recommended by Feulgen and Rossenbeck (1924). The optimum times are listed in Table 7.1. The occasional tendency of sections to come off their slides during treatment with hot acid might be avoided by using more concentrated acid at room temperature as suggested by Itikawa and Ogura (1954).

Other aldehyde reagents have been used in place of Schiff's

reagent but none offer any significant advantages and some, such as 2-hydroxy-3-naphthoic acid hydrazide (Pearse, 1951), make the method unnecessarily complicated. It is important that both the test and the control sections are not left excessively long in Schiff's reagent because the reagent, being acid, tends to cause some hydrolysis (Serra, 1943).

The importance of an unhydrolysed control section was clearly demonstrated by Bauer (1932, 1933). Schiff-positive materials might already be present in the tissue section and lead to false positive reactions in the nucleal test. Some reactive aldehydes occur naturally in tissues, such as certain lipids and constituents of elastic fibres and xylem elements in plants, or are introduced during fixation either directly, as formaldehyde, or indirectly, by hydrolysis or oxidation.

The procedure for the nucleal test is as follows:

1. Bring sections to distilled water. Hydrolyse test sections in 1N. hydrochloric acid at 60° C. for the optimum time for the particular fixation (see Table 7.1). Place control sections in distilled water at 60° C. for the same time.
2. Wash sections with water and treat with Schiff's reagent followed by sulphurous acid rinse (see page 38).
3. Counterstain cytoplasm. Light green gives good contrast. Orange G or picric acid can be used. (Do not stain nuclei with haematoxylin!)

Sites of DNA are magenta coloured.

7.4 Ribonucleic acids: the pyronin/methyl green test

The most generally used method for RNA depends upon a very non-specific property of the nucleic acids, their affinity for basic dyes. Such basiphilia is characteristic of substances having acidic groups and the nucleic acids are not, of course, the only basiphilic constituents of tissues. To establish what part of any staining with basic dyes is due to nucleic acids, appropriate extraction methods (page 120) must be applied to control sections.

The general phenomenon of basiphilia is discussed in Chapter 4 (page 52). Most basic dyes do not selectively stain nucleic acids although an exception might be methyl green which can be made quite selective for DNA. It is frequently used with pyronin, a mixture introduced by Pappenheim (1899) and modified by Unna

(1902). The Pappenheim–Unna stain in one form or another has continued to be widely used for studies on DNA (methyl green) and RNA (pyronin).

The selectivity of pyronin for RNA is generally considered to be low. It can stain depolymerized DNA and, possibly, some proteins in addition to RNA (Kurnick, 1950; Pollister & Leuchtenberger, 1949; Taft, 1951). Pyronin Y tends to be more selective for RNA than pyronin B (Taft, 1951*a*; Kurnick, 1952*a*, 1952*b*). The differential staining of DNA and RNA by a mixture of pyronin and methyl green is dependent upon the hydrogen-ion concentration of the mixture, for most tissues, the optimum being around pH 4·8.

Methyl green staining has been studied in detail by Kurnick (1950, 1950*a*; Kurnick and Mirsky, 1950) and by Pollister and Leuchtenberger (1949). They conclude that under proper conditions, methyl green can be highly specific for DNA and that the intensity of staining can be proportional to the amount of highly polymerized DNA that is present. These investigators also conclude that the specificity of the stain for DNA is probably related to the high degree of polymerization of the nucleic acid. Methyl green does not stain the much less highly polymerized RNA or depolymerized DNA. This differentiation has been explained on a stereochemical basis by Kurnick (1950) and by Pollister and Leuchtenberger (1949). There is also evidence that in many tissue constituents proteins block groups that would otherwise react with the dye (Alfert, 1952). It is important that the methyl green be purified immediately before use by chloroform extraction (Kurnick, 1950*a*; Leuchtenberger, 1950).

Jordan and Baker (1955) reinvestigated Brachet's (1953) pyronin/methyl green technique and modified it so that good results can be obtained with dyes of British manufacture. Their buffered staining solution is prepared as follows:

1. Extract freshly prepared 0·5% aqueous methyl green with chloroform at least 8 times or until all the purple impurities have been removed and the chloroform remains colourless.
2. Prepare 0·2M. acetate buffer, pH 4·8, by mixing 119 ml. 0·2M. sodium acetate and 81 ml. 0·2M. acetic acid.
3. Mix 37 ml. 0·5% aqueous pyronin G, 13 ml. extracted methyl green solution, and 50 ml. acetate buffer. This

staining mixture can be used at once and is stable for at least 4 months. With some dyes obtained in North America it has been necessary to increase the proportion of methyl green.

For the pyronin/methyl green test:

1. Bring section to water, rinse in distilled water and blot dry.
2. Stain for 30 minutes in buffered staining solution.
3. Rinse for a few seconds in distilled water and quickly blot almost dry.
4. Dehydrate for 1 minute in acetone. Pass through 1 : 1 acetone-xylene into xylene and mount in a neutral resin.

DNA is stained blue, blue-green or green. RNA and possibly other basiphilic material are stained red. Confirmation of the RNA requires control slides, preferably treated with ribonuclease (page 121) although other selective extraction methods (page 123) can be used.

A modification of the pyronin-methyl green test applicable to frozen-dried tissues has been described by Lavarack (1955).

7.5 Selective extraction methods

The procedures included here are generally referred to as extraction methods but, while some do extract nucleic acids, others simply modify the acids so that they lose their normal staining properties. The effectiveness of these methods depends upon both the tissue and its fixation. The methods can be divided into enzymic and chemical techniques.

7.51 Enzymic. The nucleases (Schmidt, 1955) can be used in histochemical studies on the nucleic acids to increase the selectivity of otherwise relatively unselective methods such as pyronin/methyl green staining as well as to test the specificity of various methods. Certain precautions should be observed when using the nucleases. Control slides should be used that have been treated with the medium alone or with the medium containing inactivated enzyme. When used in studies on new or modified techniques, additional enzyme-treated control slides should be tested by already established techniques.

7.511 Ribonuclease. The use of ribonuclease in histochemical studies of RNA with basic dyes was first described by Brachet

(1940, 1941) and has since been used by many investigators. The enzyme cleaves internucleotide bonds in two steps: (1) intermolecular transphosphorylation followed by (2) hydrolysis. This biphasic cleavage is limited to bonds between 3′-pyrimidine nucleoside phosphoryl groups and the 5′-hydroxy groups of adjacent nucleotides, whether purine or pyrimidine (Schmidt, 1955).

For many histochemical studies, a relatively crude preparation of ribonuclease (Brachet, 1941) can be used, but highly purified preparations of the enzyme are preferable for critical studies. An enzyme free of any observable activity on proteins or DNA can be prepared by McDonald's (1948) modification of Kunitz's (1940) method for crystalline ribonuclease. Commercial preparations of the enzyme can be further purified by briefly boiling with ammonium sulphate (Pollister, Himes and Ornstein, 1951). Solutions of the enzyme should be freshly prepared before use lest bacterial contamination contribute any proteolytic activity. Brachet (1953) recommends treating tissue sections for 1 hour at 37° C. with a solution containing 10 mgm. of the crystalline enzyme in 100 ml. distilled water, adjusted to pH 6. Control sections should be similarly treated with distilled water.

Following treatment with ribonuclease or the medium, the test and control sections should be stained with pyronin-methyl green. Comparing the test and control sections will show that there is complete loss of pyronin staining due to RNA. Any staining due to acid mucopolysaccharides or other basiphilic substances will be unaffected. Ribonuclease does not alter the nucleal reaction or methyl green staining of nuclei.

7.512 Deoxyribonuclease. Deoxyribonuclease depolymerizes the long-chain DNAs down to 'oligonucleotides' consisting of only a few nucleotides (Schmidt, 1955). It has been used relatively little in histochemistry, mostly in studies on chromosomes and on nuclei of liver and nerve cells. The enzyme acts only on fixed, not fresh, nuclei (Ely & Ross, 1949). Following treatment with deoxyribonuclease, DNA no longer gives a positive nucleal reaction, absorbs ultraviolet light or is stained by basic dyes (Brachet, 1947). The enzyme does not alter cytoplasmic basiphilia. An active, highly specific enzyme is difficult to prepare. McCarthy's (1946) method can be used but it might be preferred

TABLE 7.2

EXTRACTION OF NUCLEIC ACIDS

Treatment	*Time for Removal*		*References*
	RNA	*DNA*	
1N. Hydrochloric acid, 60° C.	3–10 minutes	incomplete in 50 minutes	Deane, 1946; Vendrely & Lipardy, 1946; Vendrely-Randavel, 1949; Ely & Ross, 1949, 1949*a*
5–10% Perchloric acid, 5° C.	4–18 hours		Ogur & Rosen, 1949, 1950; Erickson, Sax & Ogur, 1949
25° C.	2 hours	incomplete in 22 hours	
70° C.	20 minutes	20 minutes	Seshachar & Flick, 1949; Sulkin & Kuntz, 1950; Koenig, 1950
5% Trichloracetic acid, 90° C.	15 minutes	15 minutes	Schneider, 1945; LePage, 1951; Koenig & Stahlicker, 1951

to purchase a commercial product. Deoxyribonuclease is thermolable. It requires a bivalent cation, such as Mg^{++}, Mn^{++}, Fe^{++} or Co^{++}, as an activator and has a pH optimum between 6 and 7, depending on the cation (Miyaji & Greenstein, 1951). Some fixatives render DNA relatively resistant to DNAase (Jacobson & Webb, 1952). Satisfactory results on tissue sections can usually be obtained with a 0·01% solution of the enzyme, at pH 6 and 37° C., in 30–60 minutes.

7.52 Chemical. Several strong acids have been used to extract the nucleic acids from tissue sections. Depending upon the conditions, either RNA or both RNA and DNA can be removed. The effects of some of the acids are summarized in Table 7.2. Trichloracetic acid also removes some (8%) protein (Le Page, 1951). The time and temperature of acid extraction can be adapted to various tissues and fixatives as illustrated by the investigations of Sulkin and Kuntz (1950), Koenig and Stahlecker (1951, 1952) and Atkinson (1952, 1952*a*).

Following treatment with hot water or 5 per cent citric acid, DNA is no longer basiphilic although it still gives a positive nucleal reaction (Pollister & Leuchtenberger, 1949; Brachet, 1942). Bile salts have been used to remove RNA from bacteria (Henry & Stacey, 1943, 1946). 0·1N. potassium hydroxide, suggested by Sulkin (1951), has been used very little. Le Page (1951) has recommended hot aqueous barium perchlorate.

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CHAPTER 8

Proteins

The demonstration of proteins has not occupied a prominent place in histochemistry perhaps because proteins are usually dismissed as being constituents of practically every part of a cell or tissue. Nevertheless, it is sometimes desirable to determine whether a particular structure contains any protein and, if so, the nature of the protein.

8.11 Classification. The classes of proteins that can be recognized histochemically are listed in Table 8.1. The *simple proteins* are

TABLE 8.1

CLASSIFICATION OF THE PROTEINS

Simple proteins
Conjugated proteins
- Nucleoproteins
- Glyco- and mucoproteins
- Chromoproteins
- Lipoproteins

naturally occurring proteins that yield only α-amino acids and their derivatives upon hydrolysis.

The *conjugated proteins* can be regarded as compounds of simple proteins with non-protein constituents such as the nucleic acids in nucleoproteins, carbohydrates in glyco- and mucoproteins, coloured compounds such as haematin or melanin in chromoproteins, and simple or compound lipids in lipoproteins.

8.2 Preparation of tissues

With some histochemical methods for proteins, the choice of fixing agent is important because of its influence upon the reactivity of the tissue. Formaldehyde, for example, reacts with

α-amino groups so that formalin fixation can interfere with tests depending upon these groups. Oxidizing agents can interfere with tests for sulphydryl groups by oxidizing them to disulphides. Tissues fixed in mixtures containing chromic acid or potassium dichromate tend to be resistant to the action of proteolytic enzymes.

Ordinarily, the possible loss of proteins during fixation and tissue preparation need not be considered. It occurs to a negligible extent with neutral formalin or Carnoy's fluid (Hartlieb, Diefenbach & Sandritter, 1956). Occasionally, however, the loss of a particular protein becomes a significant problem as in the extraction of insulin by acidified ethanol.

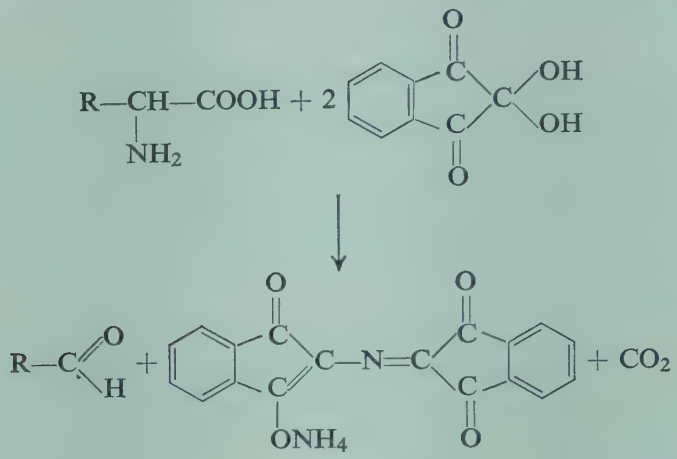
8.3 General methods

Many of the classical tests for proteins and amino acids used in biochemistry cannot be used in histochemistry because they are too destructive, inapplicable to tissue sections, or do not give reaction products that are coloured intensely enough. Most of the tests that can be used are selective or specific for certain amino acids. Although these tests are for only a part of a protein molecule, a positive reaction can be interpreted as indicating the presence of protein because of the unlikelihood of free amino acids remaining in tissue sections. The most general histochemical tests for proteins are those depending upon the reaction of free amino groups with reagents such as *p*-dimethylaminobenzaldehyde, triketohydrindene hydrate (ninhydrin), or alloxan. Some of these methods have been investigated by Burstone (1955) who concluded that certain modifications of the dinitrofluorobenzene, ninhydrin-Schiff, alloxan-Schiff, and chloramine-Schiff tests are suitable for studies on proteins. Wartenberg (1956) compared the alloxan-Schiff, ninhydrin-Schiff and *o*-diacetylbenzene (Voss, 1940) tests. He modified the *o*-diacetylbenzene test to give a more intensely coloured reaction product.

The three tests to be described below have been selected because they depend upon different reaction mechanisms and also differ in the variety of groups with which positive reactions are given.

8.31 Alloxan and ninhydrin/Schiff tests. Alloxan reacts with an α-amino acid to yield an aldehyde and a pale red coloured

product (Krasser, 1886; Hurtley & Wootton, 1911; Giroud, 1929). Similarly ninhydrin (= triketohydrindene hydrate) reacts with an α-amino acid yielding an aldehyde and a pale blue product (Ruhemann, 1910; Harding & MacLean, 1915; Berg, 1923):

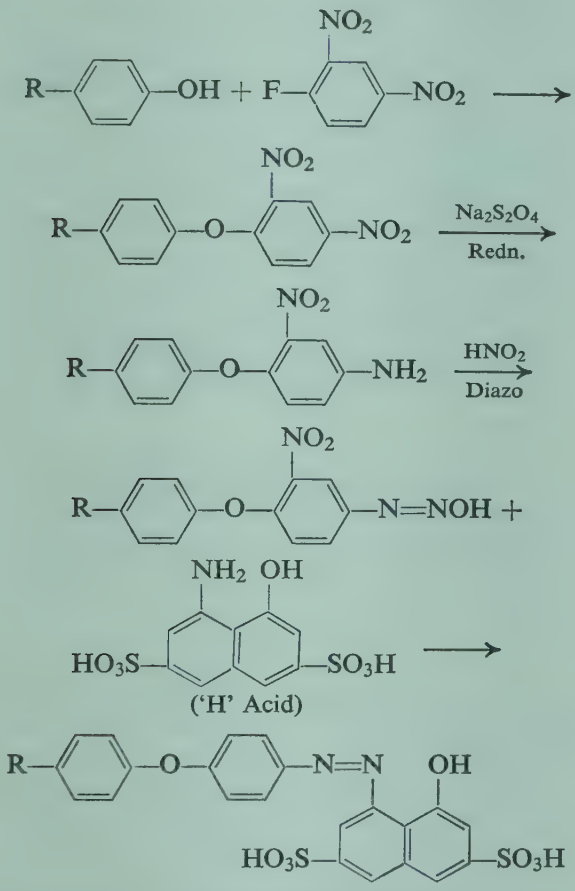


In neither case is the colour sufficiently intense to be suitable for histochemical purposes. The aldehyde, however, does not diffuse appreciably and can be demonstrated by Schiff's reagent. This forms the basis of the alloxan and ninhydrin/Schiff tests (Yasuma & Ichikawa, 1953). Both can be applied to formalin-fixed tissues.

1. Bring sections to water and place in 1% alloxan or 0·5% ninhydrin in absolute ethanol at 37° C. for 20–24 hours.
2. Rinse sections for 2 minutes in each of 3 changes of absolute ethanol, then in distilled water.
3. Continue with Schiff's test (page 37), dehydrate and mount.

8.32 Dinitrofluorobenzene test. Sanger (1945, 1949) described the use of 2,4-dinitrofluorobenzene (DNFB) for studies on amino acids in connection with his investigation of the structure of insulin. The reagent reacts not only with free α-amino groups but also with the ε-amino group of lysine, sulphydryl groups, the phenolic group of tyrosine (Sanger, 1945, 1950) and the imidazole group of histidine (Porter, 1950). Danielli (1949, 1953) was the first to use the reagent in the histochemical study of proteins, but

his method does not always give reproducible results (Pearse, 1953; Burstone, 1955). Burstone (1955) has developed a DNFB method that gives good results with all types of fixed tissue. Because the initial DNFB reaction products are colourless or only a pale yellow, they are reduced, diazotized and coupled with



1-amino-8-naphthol-3,6-disulphonic acid ('H acid') to yield deep, reddish-purple products. This method, too, can be applied to formalin-fixed tissues.

1. Prepare the DNFB reagent by dissolving 0·5 gm. 2,4-dinitrofluorobenzene in a mixture of 5 ml. 0·2N. sodium hydroxide and 95 ml. 95% ethanol.

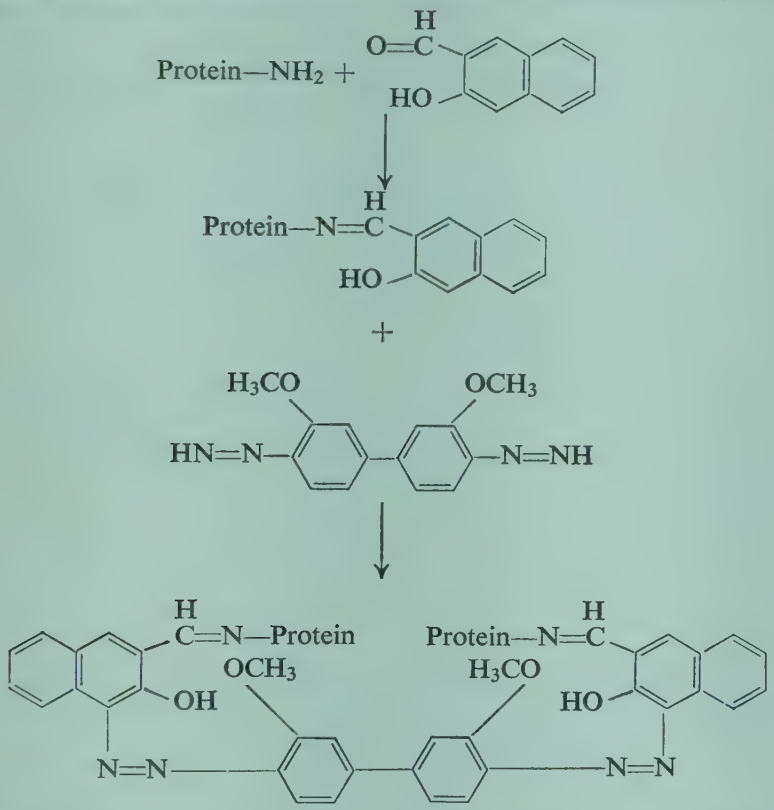
2. Bring sections to water and leave in DNFB reagent for 20–24 hours at room temperature. The sections should be yellow.
3. Rinse sections for 2 minutes in each of 3 changes of 90% ethanol, then in distilled water.
4. 5% aqueous sodium hyposulphite is used for the reduction. This can be carried out at 45° C. within 40 minutes or at 37° C. or room temperature over a longer period of time. The yellow colour should have almost or completely disappeared. Rinse sections in distilled water.
5. For diazotization, prepare the nitrous acid reagent by adding 2·5 ml. cold 4N. sulphuric acid to 50 ml. cold, freshly prepared 5% sodium nitrite. The sulphuric acid and sodium nitrite solution should be first cooled in an ice water bath. The nitrous acid should be kept in the ice water bath and used at once. If desired a small piece of ice can be added to the nitrous acid. Immerse the sections in the nitrous acid reagent at 4–5° C. for 5 minutes. Rinse slides in cold distilled water.
6. Immerse sections in a cold, saturated (about 2%) solution of H-acid (1-amino-8-naphthol-3,6-disulphonic acid) in barbitone-acetate buffer, pH 9·2, for 15 minutes.
7. Rinse sections in distilled water, dehydrate, clear, and mount in balsam or synthetic resin.

Sites of positive reactions are stained red-purple.

8.33 Hydroxynaphthaldehyde test. Weiss, Tsou and Seligman (1954) introduced a method for protein-bound amino groups based on the reaction of such groups with aldehydes to form Schiff bases. Only the α-amino groups of terminal amino acids and the δ-amino group of ornithine and the ε-amino group of lysine can at all times participate in the reaction. The aldehyde used is 3-hydroxy-2-naphthaldehyde. The pale yellow addition product is then coupled with tetrazotized diorthoanisidine,* yielding mono- and dicoupling products, or with diazotized *p*-chloro-*o*-anisidine*, yielding only one product.

* Tetrazotized diorthoanisidine is commercially available under trade names such as Naphthanil diazo blue B, Fast blue B salt, and Fast blue salt BN, and diazotized *p*-chloro-*o*-anisidine as Naphthanil diazo red RC.

For this test, tissues cannot be fixed in formalin which completely blocks the reaction. Other fixatives such as Zenker's, 80%



ethanol and acetone can be used. The tissues can be embedded in paraffin and sections cut at 5–10 μ. Sections of tissues fixed in mixtures containing mercuric chloride should *not* be treated with iodine and sodium thiosulphate.

1. Immediately before use, prepare the 3-hydroxy-2-naphthaldehyde solution by dissolving 20 mgm. of the reagent in 20 ml. acetone and adding 30 ml. 0·1M. barbitone buffer,* pH 8·5. Bring sections to water and immerse in this solution at room temperature for one hour.
2. Wash the sections for 5 minutes in each of 3 changes of distilled water.

* See Addenda on page 180.

3. Transfer sections to 50 ml. 0·1M. barbitone buffer,* pH 7·4, and add 25 mgm. tetrazotized diorthoanisidine or diazotized *p*-chloro-*o*-anisidine (footnote, page 132). Mix gently. The maximum colour develops in 4–6 minutes.
4. Wash sections in tap water and mount in Kaiser's glycerogel or dehydrate, clear and mount in synthetic resin.

With tetrazotized diorthoanisidine, there are two positive reactions. The monocoupling product is red and suggests the presence of relatively few reactive amino groups. The dicoupling product is blue and suggests more numerous amino groups. Intermediate colours are also obtained. With diazotized *p*-chloro-*o*-anisidine there is only one product, a brilliant red.

8.4 Differential methods

Biochemically, the *simple proteins* are subdivided mostly according to solubility and precipitability. Such tests have been used very little in histochemistry although some applications are described by Pearse (1953). However, some of the simple proteins have other properties that can be used as the basis of tests; for example, the histones and protamines are basic proteins and, therefore, acidophilic. The protamines also contain unusually large amounts of arginine and give strong reactions with the Millon test.

The presumptive histochemical identification of a *conjugated protein* depends upon the demonstration of protein as well as the appropriate non-protein moiety in the same site.

8.41 Selective staining. Selective staining with acidic and basic dyes under controlled conditions (page 52) is useful in some cases, such as the differentiation of acidic and basic proteins. Levine (1940) has shown that such staining depends upon protein-buffer, protein-dye, and dye-buffer interactions and, therefore, cannot be used as a direct indication of the isoelectric point of the protein. Nevertheless, several methods for the differentiation, if not identification, of proteins have been devised including the microspectrophotometric technique of Singer and Wislocki (1948) and its simplified version, the 'methylene blue extinction test' of Pearse (1949, 1950).

8.42 Proteolytic enzymes. Whereas enzymic hydrolysis is used moderately often in studies on carbohydrates and nucleic acids,

* See Addenda on page 180.

it is applied much less frequently in studies on proteins. Generally, the proteolytic enzymes are used to establish the presence or absence of protein in a given structure rather than to differentiate between classes of proteins. Pepsin and trypsin, however, do differ in their action on certain tissue components; collagen and reticulin, for example, being hydrolysed moderately readily by pepsin but not by trypsin.

Tissues should be fixed preferably in 80% ethanol or Carnoy's fluid or only briefly in alcoholic formalin. Aqueous formalin or dichromate or chromic acid mixtures should not be used. Paraffin sections should be prepared and treated as follows (Lillie, 1954):

1. Immerse test sections in 0·1% pepsin in 0·1N. hydrochloric acid, or 0·1% trypsin in 0·01M. phosphate buffer,* pH 7·6, containing 0·4% sodium chloride and 0·1% sodium fluoride. Incubate at 37° C. and remove sections at intervals from ½ to 18 hours.
2. Immerse 'solvent control' sections in the same solvent as used for the enzyme. Incubate at 37° C. until the last of the test slides has been removed.

The digested test sections should be compared with undigested controls and with the 'solvent control' sections.

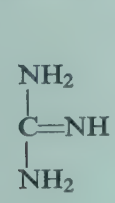
8.43 Fluorescent antibodies. The application of immunochemical reactions to the localization of specific proteins in tissue sections is one of the newest and most exciting developments in histochemistry. The technique had its practical origin in the work of Coons and his associates (1941, 1942) who developed a method of coupling antibodies with fluorescein isocyanate to give intensely fluorescent reagents for demonstrating the corresponding antigens. This specialized method has been recently reviewed by Coons (1956) and by Mellors (1957).

8.5 The Sakaguchi test for arginine

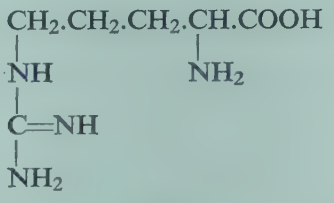
The Sakaguchi test (Sakaguchi, 1925) is specific for derivatives of guanidine (I) having at least one unsubstituted hydrogen on each of the amino groups. Arginine (II) is the only compound ordinarily found in tissue sections that meets these requirements. Histochemical adaptations of the test have been introduced by various authors (Baker, 1944, 1947; Serra, 1944, 1946; Serra &

* See Addenda on page 180.

Queiroz Lopes, 1944; Thomas, 1946, 1950; Liebman, 1951). Tissue sections are treated with an alkaline mixture of α-naphthol



I



II

and sodium hypochlorite or sodium hypobromite. A reddish colour indicates a positive reaction. The mechanism of the test is not known. Free arginine would be removed from tissues during fixation, dehydration and embedding so that in practice, the method is specific for protein-bound arginine.

A new version of the Sakaguchi test using 8-hydroxyquinoline instead of α-naphthol has been adapted for histochemical use by Warren and McManus (1951, 1951*a*) and by Carnes, Brown and Thomas (1953).

Small intestine and mature testis are suitable test tissues for the Sakaguchi reaction. The intestinal epithelial and smooth muscle cells give moderately strong reactions. The nuclei of spermatozoa react intensely.

High concentrations of arginine in animal tissues, other than fish sperm, are indicative of basic proteins such as histones. Chromosomal bands and nucleoli react positively, the former even after treatment with deoxyribonuclease (Serra & Queiroz Lopes, 1944). The Sakaguchi test is not very suitable for cytological studies, however, because thick sections are required, the reagents are rather destructive and the colour fades.

8.51 Preparation of tissues. Most of the common fixatives can be used with the Sakaguchi test: Zenker, Bouin, Susa, formaldehyde- or acetic-sublimate, or formaldehyde-saline as well as freeze-drying. The tissues can be embedded in celloidin or paraffin and cut at at least 15 μ. Paraffin sections should be covered with collodion.

8.52 Baker's method

1. To 2 ml. 1% sodium hydroxide, add 0·1 ml. 1% α-naphthol in 70% ethanol and 0·2 ml. fresh 1% sodium hypochlorite

(any fresh household bleaching solution, diluted with 5–10 parts of water depending on strength). Mix thoroughly and immediately pour over section.

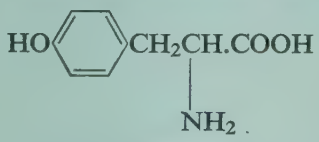
2. After 15 minutes, pour off reagent and wash section with 25% chloroform in pyridine.
3. Mount in the chloroform-pyridine mixture and examine at once.

Proteins containing arginine are stained pink or red. The colour is instable and often begins to fade within a few hours when sections are mounted in chloroform-pyridine, more rapidly in balsam.

A longer method giving more permanent preparations has been described by Liebmann and Thomas (1951).

8.6 The Millon test for tyrosine

One of the oldest tests for proteins is Millon's (1849) which, in reality, gives a positive reaction with most phenolic compounds provided the phenol is not doubly substituted in the *ortho-* and *meta*-positions. Among the amino acids, the test is specific for tyrosine. It appears that two reactions are involved in the test

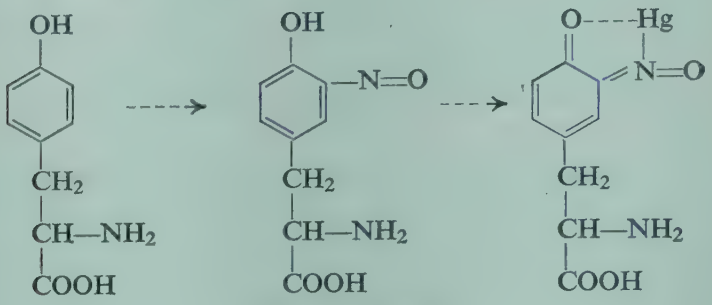


(Tyrosine)

(Vaubel, 1900; Gibbs, 1927). The phenol is first converted to a nitrosophenol which can then form intensely coloured complex salts with mercury as well as most other heavy metals (Feigl, 1940). Several histochemical adaptations of Millon's test have been published (Bensley & Gersh, 1933; Serra & Queiroz Lopes, 1945; Pollister, 1950) but the reaction product is not very intensely coloured and tends to appear transparent. A more intense colour is obtained when the mercuric sulphate reagent of Folin and Ciocalteau (1927) is used instead of Millon's reagent. Baker (1956) has recently described the following method using this reagent.

Suitable test tissues are the duodenum and adjacent pancreas where secretion granules in some cells in Brunner's glands and

zymogen granules in the acinar cells of the pancreas give very strong reactions.



(Millon's test)

8.61 Preparation of tissues. The tissues should be fixed in formaldehyde-saline and embedded in celloidin. Other fixatives such as Zenker's or Heidenhain's 'susa', but not Helly's, can be used but the reaction is less intense. Frozen or paraffin sections are not recommended because they do not withstand the reagents well.

For most purposes, cut celloidin sections at 20–30 μ and store in 70 or 80% ethanol. For special studies such as cytological investigations, thinner sections can be used but the colour will be less intense.

8.62 Procedure

1. Prepare mercuric sulphate reagent by adding 10 gms. mercuric sulphate to 100 ml. 3·6N. sulphuric acid, heating until all the salt is dissolved. Cool the solution and dilute to 200 ml. with distilled water. This reagent is stable.
2. Bring a section through 50% ethanol to distilled water.
3. To 5 ml. of the mercuric sulphate solution in a 50 ml. beaker, add 0·5 ml. freshly prepared 0·25% aqueous sodium nitrite. Put the section in this mixture and heat gently just to boiling.
4. Remove the beaker from the heat and let cool for a few minutes. Wash the section for at least 2 minutes in each of 3 portions (50 ml. or more) of distilled water.
5. Mount in glycerol or, alternatively, take through 50% glycerol and mount in Kaiser's glycerogel.

A red, pink, or yellowish-red colour constitutes a positive reaction

indicating the presence of protein-bound tyrosine. Proteins deficient in this amino acid, such as collagen and protamine, give negative reactions (Pollister, Himes & Ornstein, 1951).

8.7 Tests for sulphydryl and disulphide groups

Some proteins contain appreciable numbers of sulphydryl or disulphide groups provided by the amino acids, cysteine, cystine, and methionine. A variety of histochemical methods have been devised for demonstrating protein-bound sulphydryl groups. Generally, the demonstration of protein-bound disulphide groups depends upon applying the same tests after the disulphide groups have been reduced to sulphydryl groups.

Gomori (1956) critically studied the various methods for protein-bound sulphydryl and disulphide groups and concluded that the tetrazolium reduction and the dihydroxydinaphthyldisulphide (DDD) techniques are the most reliable. Both methods exhibit good specificity for sulphydryl groups as shown by the results following specific blockade. The tetrazolium method tends to give greater contrast while staining by the DDD technique is more diffuse. There are some differences in the relative intensities of staining of various structures by the two methods. Which method gives the truer picture cannot be decided at present. Semiquantitative visual assessment of the staining is unreliable.

Except under special circumstances it is perhaps best at present to consider the combined demonstrations of sulphydryl and disulphide groups. Sulphydryl compounds are very easily converted to disulphides by mild oxidation, even by air. At best, probably only a small proportion of the original sulphydryl will be preserved even by special fixatives and more or less of this will be oxidized during the subsequent processing and cutting of the tissue. Consequently, while a positive result is significant, a negative one does not necessarily mean that no sulphydryl groups were originally present. On the other hand, it is possible to obtain nearly complete preservation of sulphydryl plus disulphide groups which can then be demonstrated with tetrazolium or DDD following practically quantitative reduction.

8.71 Preparation of tissues

8.711 Fixing. One percent trichloracetic acid in 80% ethanol has been suggested as a fixative preserving sulphydryl groups. It

probably does not preserve all the sulphydryl groups and it is a poor fixative, lysing some cells and extracting certain proteins. Gomori (1956) recommends mercuric chloride for studies on the total sulphydryl and disulphide groups. This reagent is a better cytological fixative. With sulphydryl compounds it forms insoluble mercaptides that are subsequently oxidized by the iodine during the removal of the mercurial precipitates from the tissues. Formaldehyde is also satisfactory (Barrnett & Seligman, 1954).

8.712 Embedding and sectioning. Following fixation, the tissues can be embedded in paraffin. The sections should be brought to alcohol and coated with collodion before being taken down to water and treated with iodine. The iodine decomposes the mercaptides formed during fixation in mercuric chloride and the thiazolidine carboxylic acid formed from cysteine during fixation in formaldehyde.

8.713 Reduction of disulphide groups. For reducing the disulphide groups to sulphydryls, an alkaline solution of thioglycollic acid (Hardy, 1952; Rudall, 1952; Barrnett & Seligman, 1954) or thioglycerol (Gomori, 1956) can be used.

Thioglycollic acid method. Prepare a fresh aqueous solution (0·2–0·5M.) of thioglycollic acid. Titrate to pH 8·0 with 0·1N. sodium hydroxide. Immerse collodion-coated section for 1–2 hours in the reagent at 50° C. (Barrnett & Seligman, 1954).

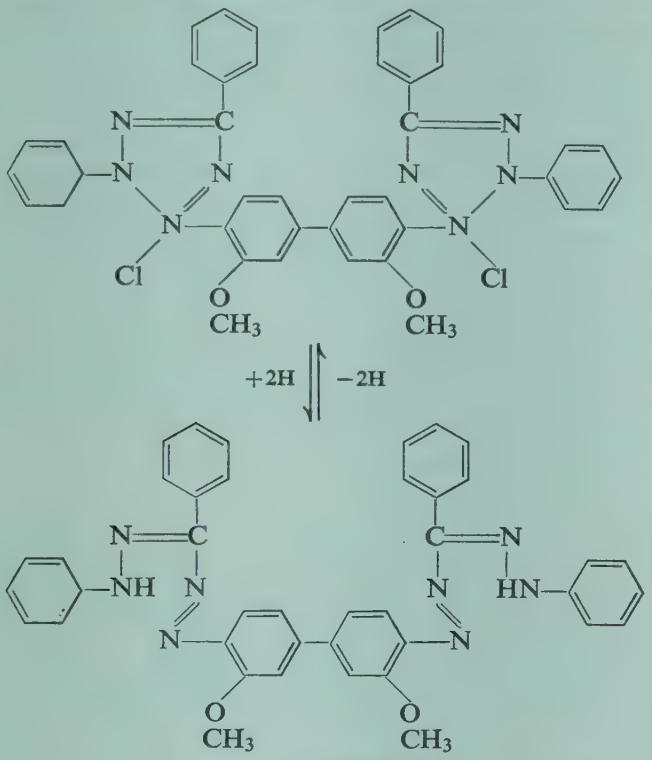
Thioglycerol method. Dissolve 3 ml. thioglycerol in 40 ml. water and add 10 ml. 0·5M. borate buffer,* pH 8·5–9·1. Immerse collodion-coated section in the mixture, overnight, at room temperature or 37° C., or for 1–2 hours at 50° C. (Gomori, 1956).

Following reduction by either reagent, the sections should be washed in several changes of large amounts of distilled water. Tap water, especially if chlorinated, should not be used because of the risk of oxidation. The sections can be kept for not more than a few hours in 0·5% acetic acid.

8.72 Tetrazolium reduction test. The histochemical test for sulphydryl groups based upon the reduction of tetrazolium salts in an alkaline medium was introduced by Barrnett and Seligman in 1952. Several variants have been suggested by other authors (Pearse, 1953*a*; Rogers, 1953; Barrnett & Seligman, 1954; Formisano & Montagna, 1954). The tetrazolium salts vary in their

* See Addenda on page 180.

ease of reduction by sulphydryl groups. Triphenyltetrazolium, for example, cannot be reduced under histochemical conditions,



(Blue Tetrazolium)

whereas, neotetrazolium can be reduced readily. Reduction is facilitated by cyanide (Findlay, 1955). Even so, some tetrazolium salts require a strongly alkaline (pH 11–12) reaction mixture, while others require only a moderately alkaline (*c.* pH 8·5) medium. The latter is preferable because there is less risk of damaging the tissue section. Findlay (1955) has shown that reduction of tetrazolium salts by sulphydryl groups under histochemical conditions is greatly influenced by such factors as the concentrations of the reactants, pH, presence of other reducing substances, and oxygenation.

Gomori (1956) has devised a method using neotetrazolium at pH 8·5–8·8 in the presence of cyanide. He has suggested that

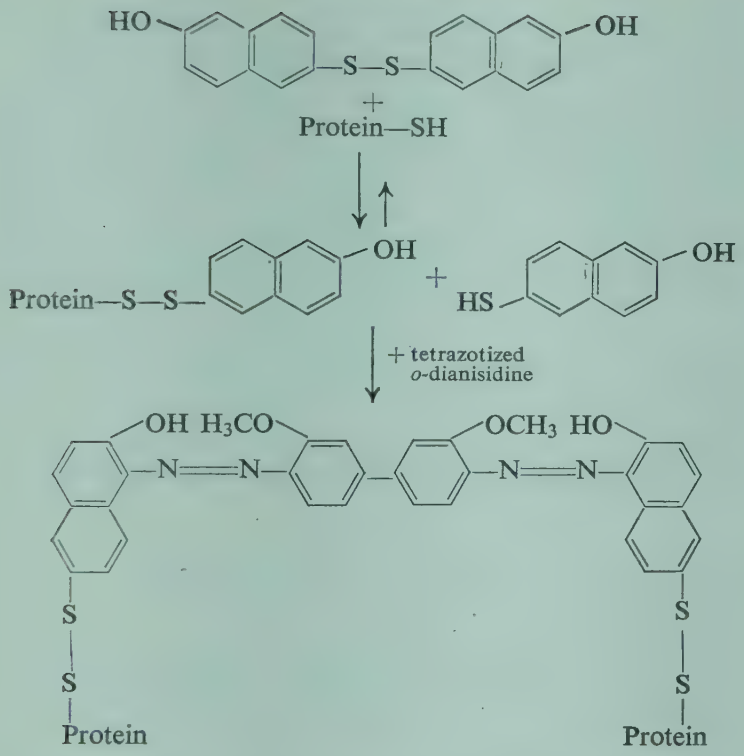
iodophenyl-nitrophenyltetrazolium could be used in the same way at pH 7·5–7·8.

1. To prepare the neotetrazolium reagent, dissolve 1 gm. sodium cyanide in 15 ml. distilled water. Add one drop of phenolphthalein indicator solution. Almost neutralize the solution with 1M. (6%) acetic acid, adding just enough (12–15 ml., usually) acid that a faint pink colour remains. Avoid adding an excess of acid. Add 20 ml. 0·5M. borate buffer,* pH 8·5–8·8. Dissolve 25 mgm. neotetrazolium chloride in 5 ml. ethanol. Add this solution to the buffered cyanide solution. This reagent mixture can be kept in the refrigerator for 2–3 weeks and used repeatedly. It should be filtered every time before use.
2. Immerse reduced section in the neotetrazolium reagent until sites of sulphydryl groups are well stained a dull reddish purple, usually about 2–3 hours.
3. Counterstain nuclei with haematoxylin, if desired.
4. Mount in glycerol jelly.

8.73 Dihydroxydinaphthyldisulphide test. The other reliable histochemical test for sulphydryl groups was also introduced by Barrnett and Seligman (1952*a*). It is a two-stage procedure depending upon the reaction of naphthols with sulphydryl compounds and the subsequent demonstration of the naphthol by azo-coupling. The naphthol derivative used is 2,2′-dihydroxy-6,6′-dinaphthyldisulphide (DDD). This is coupled with tetrazotized *o*-dianisidine or with diazotized *p*-chloro-*o*-anisidine.

1. Prepare a stock solution of the reagent by dissolving 100 mgm. DDD in 60 ml. absolute ethanol. Immediately before use, add 15 ml. stock solution to 35 ml. barbitone buffer,* pH 8·5. The final ethanol concentration (30%) is critical. The DDD should remain almost completely dissolved. Incubate section for 1 hour at 56° C. in the buffered mixture, then allow section and reagent to cool for 10 minutes at room temperature.
2. Rinse section briefly in distilled water and wash for 5 minutes in each of two changes of distilled water acidified to about pH 4 with acetic acid. This converts the sodium salts of the excess reagent and the reaction by-product to the free

* See Addenda on page 180.



naphthols for extraction by the organic solvents in the next step.

3. Take section through graded alcohols, wash in absolute ether for 5 minutes, and return through graded alcohols to distilled water.
4. The diazo reagent must be freshly prepared by dissolving 50 mgm. diazotized *p*-chloro-*o*-anisidine (footnote, page 132) in 50 ml. phosphate buffer, pH 7·4. Use at once. Immerse section in reagent for 2 minutes at room temperature.
5. Wash in running tap water and mount in glycerol or dehydrate in acetone and mount in clarite.

In keeping with Gomori's (1956) recommendation, diazotized *p*-chloro-*o*-anisidine has been used in the above procedure. Tetrazotized diorthoanisidine can be used in the same way. Barrnett and Seligman (1952*a*) recommended the latter on the basis that monocoupling, yielding a pink or red product, would

indicate widely separated sulphydryl groups while dicoupling, blue, would indicate a greater concentration of the groups.

8.74 Blocking methods. Blocking procedures can be used to establish the specificity of the reactions obtained with the tetrazolium reduction and DDD methods. Iodoacetate and N-ethyl maleimide (Dickens, 1933; Rapkine, 1933; Friedmann, Marrian & Simon-Reuss, 1949) are the most reliable reagents for histochemical use. Blockade by these reagents can be reversed by exposure to alkaline cyanide solutions for 90 minutes or longer (Gomori, 1956).

N-ethyl maleimide: Incubate section for 4 hours at 37° C. in 0·1M. N-ethyl maleimide in phosphate buffer,* pH 7·4.

Iodoacetate: Incubate section for 20 hours at 37° C. in 0·1M. iodoacetic acid brought to pH 8·0 with sodium hydroxide.

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CHAPTER 9

Calcium and Iron

9.1 Inorganic constituents of tissues

From time to time methods have been published purporting to demonstrate sites of various inorganic constituents in tissue sections. Some of these are based on well-established chemical reactions that are also reliable under histochemical conditions. Others are so unsoundly based and so unreliable that they do not warrant any serious consideration. While there are methods for various inorganic anions and cations (see Bibliography) only those for calcium and iron will be considered in this chapter.

9.11 Classification. From a histochemical point of view, there are three main classes of inorganic constituents of tissues:

(*a*) *Soluble, reactive constituents*, including the salts in the extracellular and intracellular fluids. These substances are generally completely ionized and freely diffusible. Because of their ready mobility, their localization is difficult, if not impossible, to maintain during fixation and testing. Freeze-drying is the most promising method for preparation of the tissues. While the use of non-aqueous solutions of reagents possibly diminishes diffusion during testing, appreciable displacement can still occur. There are at present no truly practical methods for the histochemical demonstration of this class of inorganic anions and cations in tissue sections.

(*b*) *Insoluble, readily reactive constituents*, including those substances which, even though insoluble, react either directly or, at most, after treatment with dilute acid. Maintenance of their localization usually presents no problems during fixation provided the fixative mixture does not dissolve the constituent. When treatment with acid is necessary, the test reagent must be in the tissue from the very beginning of such treatment, as illustrated by Perls' test for iron (page 158).

(*c*) *Insoluble, non-reactive constituents* are those often described as 'masked', to use Molisch's (1893) expression, because they are incorporated into organic complexes which are either insoluble or only slowly diffusible and unreactive. Preservation of localization usually presents no problem. Demonstration of the inorganic constituent, however, requires that it be 'unmasked', sometimes by strong reagents or heat often resulting in undesirable destruction of the tissue section.

9.12 Types of methods. For the investigation of the inorganic constituents in tissue sections, three types of techniques are available:

(*a*) colour reactions using inorganic or organic reagents,
(*b*) radioautography, and
(*c*) microincineration.

Only the first type will be considered here.

Radioautography is discussed in a recent review by Fitzgerald (1955) and in the book by Boyd (1955). The problems of terminology and the use of 'autoradiography' or 'radioautography' have been discussed at some length by Joftes (1957), Preuss (1957) and their associates. Microincineration has been reviewed by Glick (1949), and by Horning (1951). It is discussed in detail in the book by Hintzche (1956).

9.2 Calcium *

9.21 Methods for calcium. Calcium salts occur in tissues in all three states of histochemical reactivity (page 148). There are no methods of established reliability for demonstrating *soluble* calcium salts in tissue sections. Localization is poor or misleading when a fixative containing formalin and oxalate (Rabl, 1923) is used (McGee-Russell, unpublished).

There are two classes of methods for demonstrating deposits of *insoluble, reactive* calcium salts in tissue sections:

(*a*) metal substitution tests, and
(*b*) dye-lake tests.

* This section has been prepared largely from unpublished material by Dr. S. M. McGee-Russell of Birkbeck College, London, who very kindly provided me with the detailed results of his investigations.
(**N.B.** see *J. Histochem. & Cytochem.* **6**, 22 (1958)).

As is shown in Table 9.1, deposits containing calcium carbonates or phosphates can be demonstrated quite readily by metal-substitution and dye-lake methods whereas only one test in each class gives a positive result with calcium oxalate.

TABLE 9.1

REACTIONS WITH INSOLUBLE CALCIUM SALTS

Class	*Reagent*	*Carbonate*	*Phosphate*	*Oxalate*
Metal substitution	Silver	+	+	+
	Cobalt	+	+	0
	Iron (II or III)	+	+	0
	Copper	+	+	0
Dye lake	Quinalizarin	+	+	+
	Haematoxylin	+	+	0
	Alizarin red S	+	+	0
	Nuclear fast red	+	+	0

Unreactive or masked calcium can be demonstrated only by microincineration. There are no reliable unmasking procedures for calcium.

The various calcium compounds that can be found in animal tissue and the physical and chemical properties by which these can be differentiated have been reviewed by Prenant (1927). The use of certain tissues for the assessment of histochemical techniques for calcium has been described by McGee-Russell (1957).

9.22 Preparation of tissues. Tissues containing deposits of insoluble calcium salts must be fixed in neutral solutions such as neutral formaldehyde or alcohol. For fixing invertebrate tissues at least, 1 : 1 absolute ethanol and neutral or slightly alkaline formalin gives good results. Acidic mixtures cannot be used because they dissolve 'insoluble' calcium salts such as the carbonates and phosphates.

Following fixation, the tissues can be dehydrated, cleared and embedded as usual, but any accidental contact with acids must be avoided.

9.23 Metal substitution methods. The metal substitution tests depend upon the replacement of calcium by another metal whose localization is then demonstrated by an appropriate colour reac-

tion. The metal substitution methods vary in the amount of detail provided regarding the distribution of calcium in tissue sections and within cells. It is often possible to obtain more precise information by applying several of these methods to successive sections. However, all of these methods depend upon the same reaction mechanism so that the results are no more valid using several tests.

Of the metal substitution techniques (Table 9.2), von Kossa's silver method is the best known and the only one giving a positive reaction with calcium oxalate. Here the calcium is replaced by silver forming silver carbonate or phosphate, for example. The silver salt is then reduced to metallic silver so that the sites of the former calcium salt are blackened:

$$CaCO_3 + 2Ag^+ \longrightarrow Ag_2CO_3 + Ca^{++}$$
$$Ag_2CO_3 + 2H^+ \longrightarrow 2Ag + H_2O + CO_2$$

Other metal substitution methods that can be used to advantage in special studies are also outlined in Table 9.2. The equations for the reactions are given below. Some of these, notably cobalt and copper, tend to give appreciable non-specific background colora-

$$3CaCO_3 + 2Fe^{+++} \longrightarrow Fe_2(CO_3)_3 + 3Ca^{++}$$
$$2Fe_2(CO_3)_3 + 3K_4[Fe(CN)_6] \longrightarrow Fe_4[Fe(CN)_6]_3 + 6K_2CO_3$$

(Iron (III) substitution)

$$CaCO_3 + Cu^{++} \longrightarrow CuCO_3 + Ca^{++}$$

$$CuCO_3 + H_2N{-}\underset{\underset{S}{\|}}{C}{-}\underset{\underset{S}{\|}}{C}{-}NH_2 \longrightarrow HN{=}C{-}\!\!-\!\!-{-}C{=}NH + H_2O + CO_2$$

(Copper substitution)

tion, so that acid-treated controls should be used. The two iron methods differ in the readiness with which they demonstrate some calcium deposits so that it is advisable to try both the ferric and the ferrous techniques. Even though a lead substitution method is used in the Gomori method for phosphomonesterase II, it has

TABLE 9.2

METAL SUBSTITUTION TESTS FOR CALCIUM

Metal		Substitution Reagent		Colour Reactions		Counterstains*	
Ag	Remove paraffin, bring to water, and rinse thoroughly in distilled water	5% aq. $AgNO_3$ > 10 mins.	Rinse thoroughly in distilled water	0·5% aq. hydroquinone, 2 mins. Rinse in distilled water. 2% aq. sodium thiosulphate, 5 mins.	Rinse thoroughly in distilled water	Safranin Tartrazine Neutral red	Dehydrate, clear, and mount in balsam
Co		5% aq. $Co(NO_3)_2$ 5 mins.		Yellow ammonium sulphide (diluted 1 : 4 with water, if desired), 30 sec.		Toluidine blue Eosin Safranin	
Fe^{III}		5% aq. $FeCl_3$ 10 sec.**		5% potassium ferrocyanide freshly acidified with HCl, 1–5 mins.		Eosin Safranin Xylidine red	
Fe^{II}		5% aq. $FeSO_4$ 5 mins.		5% potassium ferricyanide, freshly acidified with HCl, 1–5 mins.		Eosin Safranin Xylidine red	
Cu		5% aq. $CuSO_4$ 5 mins.		Saturated solution of dithio-oxamide (rubeanic acid) in 70% ethanol, containing 2 drops conc. NH_4OH per 100 ml., 1 min. Rinse in 50% ethanol		No	

* Sections should be studied carefully before counterstaining in case the staining obscures some deposits or causes dislocation.

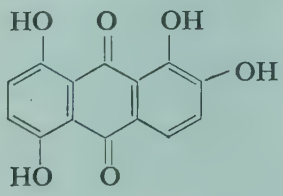
** Timing important.

not been included here because it gives less satisfactory results and the preparations are less permanent.

The metal substitution techniques have three important disadvantages. First, they really depend upon the anions in the calcareous deposits. Fortunately, from the practical point of view, in animal tissues nearly all insoluble calcium deposits consist of calcium phosphates, carbonates and, sometimes oxalates, and conversely, nearly all insoluble deposits of these anions occur as calcium salts. This is, however, not so generally true of plant materials. Second, deposits of calcium salts are not the only sites with an affinity for the metals. As a result, there is appreciable background staining. Wigglesworth's (1952) contribution on iron staining reactions and Hale's test for acid mucopolysaccharides (page 103) should be recalled in this regard. Third, there is poor penetration of dense granules and spicules.

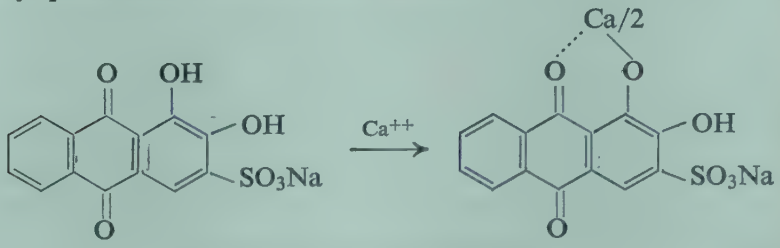
For each of the metal substitution methods outlined in Table 9.2 the paraffin should be removed and the section brought through graded alcohols to water before being immersed in the substitution reagent. It is important to remove the excess of this reagent before carrying out the second part of the test that converts the initial reaction product into the characteristically and intensely coloured final product. As noted in the table, the sections should be examined before counterstaining because there are possibilities of either changing the distribution of the final product or obscuring weakly positive sites during counterstaining.

9.24 Dye lake methods. A number of dyes have been suggested for the histochemical demonstration of calcium in tissue sections by forming intensely coloured lakes. Both the haematoxylin and quinalizarin tests give such excellent results that their use is recommended despite the disadvantage that the preparations are not permanent and must be examined at once. Permanent slides can be prepared using nuclear fast red (McGee-Russell, 1955) or



(Quinalizarin)

sodium alizarin sulphonate but the localization is subject to some diffusion error and staining must be controlled microscopically. All four reagents react with calcium carbonates and phosphates, only quinalizarin with calcium oxalate.



(Sodium alizarin sulphonate)

The formation of dye lakes with calcium salts must not be confused with the staining of calcified structures by preformed haematoxylin lakes such as the haemalum and chrome haematoxylin solutions used in histology. Such staining of tissue sections does not depend solely upon the presence of calcium salts but largely upon the surrounding organic matrix, in bone at least. Moreover, the haematoxylin lakes do not stain calcium phosphate or calcium carbonate (Schuscik, 1920; Cameron, 1930).

9.241 Haematoxylin method

1. Bring section to water and cover with 5% haematoxylin in concentrated ammonium hydroxide for 15 minutes. Add more reagent if necessary.
2. Pour off reagent, rinse with concentrated ammonium hydroxide.
3. Mount in concentrated ammonium hydroxide and examine at once.

Calcium deposits are stained blue or dark blue: calcium phosphates in 5–10 seconds, calcium carbonates in 10–15 minutes. Calcium oxalate is not stained.

The colour will diffuse immediately if sections are rinsed with anything but concentrated ammonium hydroxide. Mordant staining effects can occur on standing.

9.242 Quinalizarin method

1. Bring sections to 50% ethanol and rinse briefly in distilled water. The sections can be protected with collodion.

2. Cover section with 10% quinalizarin in 15% potassium hydroxide for from 5 seconds to 3 minutes. The longer staining time is required only for very insoluble salts.
3. Shake off stain, rinse gently in distilled water and mount in water.

Calcium deposits are stained an intense purplish-blue, cytoplasm, nuclei, connective tissue fibres, lighter shades of blue.

9.243 Nuclear fast red method

1. The commercial dye, Nuclear Fast Red, should be partially purified by extracting 2 gms. with two 100 ml. portions of distilled water. Prepare a saturated (about 0·25%) solution of the residue in distilled water.
2. Bring the section to 50% alcohol and rinse.
3. The reaction must be carried out under the staining microscope. Cover the section with the reagent and view the section through the stain. Within from 30 seconds to 5 minutes sites of calcium will become covered and surrounded by orange-red, birefringent precipitates of the calcium lake, usually best observed in sites with a reasonable amount of calcium salt.
4. When the coloured deposit is heavy, but not too diffuse, shake the stain off the slide and blot the section carefully with filter paper.
5. Immediately immerse the slide in acetone for 10–20 seconds, then in 1 : 1 acetone and xylene for 10–20 seconds. Do not counterstain. Clear in xylene and mount in balsam or synthetic medium.

A faint pink background coloration of most tissue elements cannot be confused with the positive reaction given by calcium deposits.

9.244 Sodium alizarin sulphonate method

1. Prepare the sodium alizarin sulphonate reagent by dissolving 2 gms. of sodium alizarin sulphonate in 100 ml. distilled water. Dilute 0·5 ml. concentrated ammonium hydroxide to 100 ml. with distilled water. Add sufficient of this dilute ammonia to the alizarin red S solution to bring it to pH 4·1–4·3. Usually 20–40 ml. is required, the exact volume

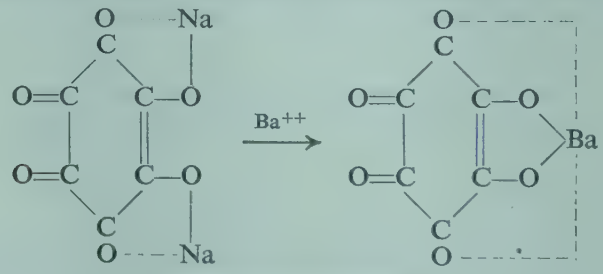
varying with the sample of the dyestuff, and the exact concentration of the ammonia solution. The pH adjustment is critical. The staining solution should be a deep iodine colour. It will be a darker colour if the pH is too high. This reagent is stable and can be used for at least a year.

2. The staining procedure is the same as that given above for nuclear fast red.

9.245 Other reagents. Other reagents forming lakes with calcium have been suggested. Purpurin and purpurin-3-carboxylic acid can be used in ammoniacal solutions. They are useful for comparison with alizarin red S because the laking is carried out in an alkaline rather than an acid medium. Unfortunately, staining with either reagent cannot be followed microscopically and the lake does not withstand dehydrating and mounting well.

Gallamine (Stock, 1949) appears to be a wholly unreliable reagent. It fails to stain some deposits that give intense reactions with other methods for calcium. Moreover, positive reactions seem to depend upon the nature of other tissue constituents in the vicinity of the calcium deposits and not upon the calcium alone.

Sodium rhodizonate (Waterhouse, 1951), a reagent for barium and strontium, as well as certain other metals, gives exceptionally good localization of calcareous deposits provided they contain even traces of these alkaline earths, as is usually the case. Sections should be brought to distilled water and immersed for one hour in a freshly prepared, saturated aqueous solution of the reagent.



(Sodium rhodizonate)

The sections can then be rinsed, dehydrated and mounted as usual. Deposits of barium and strontium are coloured red-brown, as are mercury (I), cadmium, and bismuth (Feigl, 1943).

Barium rhodizonate can be distinguished from strontium rhodizonate by solubility tests: the barium salt being insoluble in dilute hydrochloric acid and soluble in dilute potassium chromate whereas the strontium salt is soluble in the acid and insoluble in the chromate solution.

9.3 Iron

9.31 Methods for iron. In so far as the histochemical demonstration of iron is concerned, this metal appears to occur in tissues in only the ferric state. There are both reactive and non-reactive or masked types of insoluble iron compounds, exemplified by haemosiderin and haemoglobin respectively. Unlike calcium there is at least one moderately satisfactory procedure for unmasking iron in tissue sections. Gomori (1936), Lillie (1939) and Bunting (1949, 1951) have thoroughly investigated the various histochemical methods for iron.

One of the oldest histochemical tests for iron remains the most satisfactory. It is the adaptation of the Prussian blue reaction

$$4Fe^{+++} + 3K_4[Fe(CN)_6] \longrightarrow Fe_4[Fe(CN)_6]_3 + 12K^+$$

introduced by Perls in 1867. An older test that is still used is the ammonium sulphide test, usually attributed to Quincke (1880, 1896) but apparently first used in 1845 by Vogel (Mann, 1902).

$$Fe^{+++} \xrightarrow{(NH_4)_2S_x} FeS$$

It is favoured by some because the ferrous sulphide formed can be easily seen since it is black and relatively opaque while the blue ferric ferrocyanide appears rather transparent. This difference in optical properties may have led to the early impression that the sulphide test is more sensitive. Whereas the Prussian blue reaction is specific for ferric iron, other metals besides iron can yield dark brown or black sulphides with ammonium sulphide. The sulphide test has several additional disadvantages. Ferrous sulphide dissolves in acidic solutions, even in stains such as alum carmine. The test cannot be applied to tissues fixed in mixtures containing mercuric chloride because enough mercury remains after treatment with iodine to yield interfering black sulphides.

Tirmann (1898) suggested applying the sulphide test followed

by acidified potassium ferricyanide so that the final reaction product is Turnbull's blue. From the chemical point of view the

$$Fe^{+++} \xrightarrow{(NH_4)_2S_x} FeS$$

$$3FeS + 2K_3[Fe(CN)_6] \longrightarrow Fe_3[Fe(CN)_6]_2 + 3K_2S$$

method offers no advantages over the Prussian blue test. Moreover, it fails to demonstrate all the iron present in a section (Lillie, 1949) and produces some misleading artifacts—as compared with the results after the Prussian blue test, particles of iron-containing pigment appear larger and quite distorted after the sulphide test and especially Tirmann's method (Gomori, 1936; Bunting, 1949).

9.32 Preparation of tissues. To avoid artifacts, all reagents used in preparing the tissues and in testing for iron must be free of iron, preferably of analytical reagent grade. Tap water and even slightly rusty equipment should be avoided.

Neutral fixatives should be used. Buffered neutral 10% formalin preserves more iron with less diffusion than other reagents including ethanol. Acidic fixatives must not be used as diffusion takes place very readily in even mildly acidic mixtures such as Bouin's fluid. Such diffusion can lead to false localization because certain structures such as nuclei and collagen fibres have high affinities for even traces of ferric iron. No special precautions, other than iron-free reagents, are necessary during the further preparation of the tissues. The microtome knife should be free of any rust and preferably polished.

9.33 Prussian blue test. The Prussian blue test should be carried out essentially as was recommended originally by Perls (1867); that is by treating the sections first with dilute potassium ferrocyanide and then with dilute hydrochloric acid. The purpose of the acid is to ionize some of the iron in the insoluble iron compound. Provided ferrocyanide ions are also present, Prussian blue will be precipitated with virtually no diffusion of the ferric ions beforehand. Ferrocyanide ions, however, diffuse relatively slowly in tissue sections and, therefore, must be given time to reach the reaction sites before the rapidly diffusing acid. This can be achieved as Perls did or by later adding the acid to the ferrocyanide solution.

1. Bring sections to water and immerse in freshly prepared and filtered 2% potassium ferrocyanide for 5 minutes.
2. Add an equal volume of 0·2N. hydrochloric acid to the ferrocyanide solution, mix well, and leave slides in the mixture for an additional 20 minutes.
3. Wash in distilled water.
4. Stain nuclei with safranine or neutral red.
5. Dehydrate, clear and mount in a synthetic medium.

Sites of reactive iron are blue. The Prussian blue fades slowly in Canada balsam but is more stable in synthetic resins. Cytoplasmic counterstains are inadvisable because they obscure weakly positive reactions.

9.34 Unmasking with hydrogen peroxide. The iron in substances such as haemoglobin and malaria pigment cannot be demonstrated directly by the Prussian blue test. Before a positive result can be obtained, the iron must be 'unmasked' by destroying the organic part of the molecule. The most satisfactory reagent is hydrogen peroxide, introduced by Brown (1911). Following treatment with hydrogen peroxide, haemoglobin, for example, gives a moderately intense Prussian blue reaction. The iron is oxidized to insoluble, non-diffusing ferric oxide which can be detected with acidified ferrocyanide. It is doubtful whether all the iron in a 'masked' compound is rendered reactive. Moreover, it has yet to be established whether this method unmasks the iron in all materials containing masked iron. Thus a negative reaction following treatment with hydrogen peroxide must be interpreted cautiously and other techniques such as microincineration (Policard, 1934) should be considered.

1. Bring section to water and immerse in 30% (= 100 volumes) hydrogen peroxide, made alkaline with a few drops of sodium carbonate, for 30 minutes or longer.
2. Wash in distilled water.
3. Apply Prussian blue test.

Sites of masked iron give a positive Prussian blue reaction after treatment with hydrogen peroxide but not before.

Mineral acids, diluted with ethanol, and ammonium sulphide are reputed to unmask iron. However, they fail to do so with many pigments known to contain this metal. In addition, the

acids cause serious diffusion of any already reactive iron in the tissues. Chlorine and bromine have been suggested (Klein, 1929) but they are inconvenient to use and yield readily soluble, diffusible ferric halides, so that localization is poor.

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CHAPTER 10

Enzymes

The identification and localization of enzymes in cells and tissue sections is one of the newest and most rapidly developing branches of microscopical histochemistry. While some staining methods in use for 50 years or more have been alleged to depend upon enzymatic activity, such claims have been largely disproven or not yet positively established. The introduction of a method for alkaline phosphatase independently by Takamatsu (1938, 1939) and Gomori (1939) is generally regarded as the beginning of modern enzyme histochemistry. This rapidly developing field has become so large and specialized that only a survey will be attempted in this chapter. Additional details can be found in the books by Gomori (1952), Lison (1953) and Pearse (1953), and in the review by Novikoff (1955). The methods of enzyme histochemistry are more exacting and subject to more varied sources of error than other histochemical techniques. Some of these are discussed in detail by Gomori (1952). It is strongly recommended that the original literature be consulted for full details of each method, the sources of errors and special precautions.

Not all enzymes can be studied histochemically. Instead, only those enzymes that withstand tissue preparation and catalyse reactions yielding products visible microscopically can be investigated. Generally, the methods for enzymes differ fundamentally from most other histochemical techniques. In the case of most tissue constituents, the reagent reacts with the constituent so that the product is more or less directly derived from the constituent under investigation. With enzymes, however, the reagent is a substrate that is acted upon by the enzyme so that the final product is derived from the substrate alone, through some change catalysed by the enzyme.

The problems of accurate localization are great in enzyme

histochemistry. Occasionally the product of the enzyme-catalysed reaction can be seen directly but quite often it has to be subjected to further chemical reactions before it is visible under the microscope. This is well illustrated by one of the methods for alkaline phosphatase or phosphomonoesterase I. Here the substrate is usually glycerophosphate. As a result of enzyme action, phosphate ions are liberated. These are precipitated as calcium phosphate. Then, the calcium phosphate is converted to cobalt phosphate. Finally, the cobalt phosphate is converted to brownish black cobalt sulphide. At every step, there are dangers of diffusion and adsorption of the reaction products resulting in localizations not the same as the sites of the enzyme, especially at the intracellular level.

Some of the general problems of the histochemical localization of enzymes, including preparation, characteristics of the chemical reaction, properties of the substrate and the validity of localization, were well described by Nachlas in his contribution to the Symposium on Localization in Histochemistry sponsored by the Histochemical Society (1957). Earlier, Nachlas, Prinn and Seligman (1956) demonstrated the importance of the occurrence of varying proportions of lyo- and desmoenzymes in the interpretation of histochemical studies on enzymes especially with regard to the concept of localization.

10.1 Preparation of tissues

Many enzymes survive the fixing, dehydrating and embedding of tissues sufficiently to give an observable histochemical reaction in some sites. A few studies have been carried out biochemically estimating the residual enzyme activity following various steps in the preparation of the tissue, usually liver. The results summarized in Table 10.1 show that the enzymes vary widely in their resistance to the reagents used in fixing and preparing the tissues and, moreover, that with any given technique, the residual activity of a given enzyme can vary considerably from one time to the next.

For the histochemical study of some enzymes, it is necessary to use unfixed tissues. In such cases, the rapid frozen-sectioning techniques of Schultz-Brauns (1931) and Adamstone and Taylor (1948) should be considered. Diffusion of the enzyme becomes an especially important problem when fresh-frozen or briefly fixed tissues must be used. This can be overcome to some extent

TABLE 10.1

EFFECTS OF TISSUE PREPARATION ON ENZYME ACTIVITY

Enzyme	Preparation	Activity after Fixation	Embedding	Activity after Embedding	Ref.*
Acid phosphatase	Cold acetone, Absol., 7 hrs.	28%			
	28 hrs.	20%			
	50 hrs.	22%	2 hrs. 56° C.	4%	3
	72 hrs.	40%			
	72 hrs.	25%	1 hr. 60° C.	15%	2
	72 hrs.	49%	½ hr. 60° C.	11%	6
			20–40′ 56° C.	41%	8
	Cold ethanol, 80% 7 hrs.	29%			
	28 hrs.	18%			
	50 hrs.	20%	2 hrs. 56° C.	7%	3
	72 hrs.	26%			
	80% or Abs. 24 hrs.	2%			2
	10% Formalin, pH 7				
	2 hrs. 4° C.	79%			7
	2 hrs. 25° C.	40%			
	2 hrs. 37° C.	27%			
	24 hrs. 4° C.	55%			
	48 hrs. 4° C.	21%			
	10% Formal.-saline				
	14 hrs.	30%			
	41 hrs.	20%			1

TABLE 10.1 *continued*

EFFECTS OF TISSUE PREPARATION ON ENZYME ACTIVITY

Enzyme	Preparation	Activity after Fixation	Embedding	Activity after Embedding	Ref.*
	Freeze-drying	85%			2
		92%	½ hr. 60° C.	13%	6
Alkaline phosphatase	Cold acetone, Absol., 28 hrs. 50 hrs. 72 hrs.	71% 65% 64%	2 hrs. 56° C.	29%	3
	72 hrs.	49%	½ hr. 60° C.	26%	6
			20–40′ 56° C.	58%	8
	80% or Abs., 24 hrs.	75%	1 hr. 60° C.	28%	2
	Cold ethanol, 80% 28 hrs. 50 hrs. 72 hrs.	74% 88% 91%	2 hrs. 56° C.	20%	3
	Absol., 24 hrs. 80%	75% 65%	1 hr. 60° C.	40%	2
	10% Formalin, pH 7 2 hrs. 4° C. 2 hrs. 25° C. 2 hrs. 37° C. 24 hrs. 4° C.	 73% 35% 13% 26%			7
	10% Formal.-saline 2 hrs. 48 hrs.	9–23% 0–24%			1

TABLE 10.1 *continued*

EFFECTS OF TISSUE PREPARATION ON ENZYME ACTIVITY

Enzyme	Preparation	Activity after Fixation	Embedding	Activity after Embedding	Ref.*
	Freeze-drying	85%			2
		87%	½ hr. 60° C.	29%	6
'5-Adenylic acid phosphatase'	Cold acetone,		20–40′, 56° C.	47%	8
Esterase	Cold acetone, 24 hrs. 48 hrs.	78% 50%	3 hrs. 56° C.	42%	4
	72 hrs.	26%	½ hr. 60° C.	11%	6
	Cold ethanol, 24 hrs.	0%			4
	10% Formalin, 2 hrs. 4° C. 2 hrs. 25° C. 2 hrs. 37° C. 24 hrs. 4° C. 48 hrs. 4° C.	 82% 53% 23% 35% 50%			7
	Freeze-drying	91%	½ hr. 60° C.	10%	6
β-Glucuronidase	10% Formalin, 2 hrs. 4° C. 2 hrs. 25° C. 2 hrs. 37° C. 24 hrs. 4° C. 48 hrs. 4° C.	 86% 89% 91% 87% 83%			7

TABLE 10.1 *continued*

EFFECTS OF TISSUE PREPARATION ON ENZYME ACTIVITY

Enzyme	Preparation	Activity after Fixation	Embedding	Activity after Embedding	Ref.*
Sulphatase	10% Formalin, pH 7				
	2 hrs. 4° C.	74%			
	2 hrs. 25° C.	61%			
	2 hrs. 37° C.	41%			7
	24 hrs. 4° C.	58%			
	48 hrs. 4° C.	12%			
Cytochrome oxidase	Freeze-drying	56%	½ hr. 60° C.	0%	6
Succin-oxidase	Freeze-drying	37%	½ hr. 60° C.	0%	6

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by making the medium hypertonic. From 3·0 to 5·8% sodium chloride has been used by Rutenberg and his associates (Rutenberg, Cohen & Seligman, 1952; Rutenberg, Cohen, Tsou & Seligman, 1952) and up to 24 or 28% sodium sulphate by Koelle (1951).

Many enzymes are reasonably resistant to fixation. Ice-cold acetone or ethanol has been used extensively. Tissues fixed in either reagent should be embedded in paraffin. Greater care must be taken than in ordinary histological embedding with regard to times and temperatures. During storage of the paraffin blocks,

the activities of at least some enzymes remain essentially unchanged for many months. Paraffin sections can be cut, floated on water, mounted on slides with albumin, and dried as usual. Gomori (1952) recommends placing the slides for 5–10 minutes in the paraffin oven to melt the paraffin. This provides a protective coating for the tissue section so that there is no decrease in enzyme activity during storage of the sections. Before use such slides can be taken through xylene and graded alcohols. In some instances, it is desirable to coat the section with collodion (page 94) provided it is permeable to the substrate to be used.

Seligman, Chauncey and Nachlas (1951) have demonstrated that ice-cold 10% formalin can be used for many enzymes. In this case frozen, rather than paraffin, sections should be used.

10.2 Phosphomonoesterases

10.21 Phosphomonoesterase I (alkaline phosphatase). 'Phosphomonoesterase I' is the name recommended by Roche (1950) for the enzyme hydrolysing a wide variety of orthophosphate esters in an *alkaline* medium. This enzyme is specific for a particular type of bond rather than for a particular substrate. It is commonly called 'alkaline phosphatase' but this term has been used by some authors for any phosphatase having its optimum hydrogen-ion concentration in the alkaline range. For this reason, the use of 'phosphomonoesterase I' is recommended to avoid confusion. Evidence that several forms of this enzyme exist is still inconclusive (Cloetens, 1939; Gomori, 1950; Roche, 1950; Emery & Dounce, 1955; Burgos, Deane & Karnovsky, 1955). In most tissues, the optimum for the enzyme is between pH 9·2 and pH 9·6. Phosphomonoesterase I is activated by magnesium and certain other divalent cations (Freiman, 1956).

There are two general methods for the histochemical demonstration of phosphomonoesterase I. One is the method introduced by Gomori (1939) and Takamatsu (1939) using sodium glycerophosphate as the substrate. It is discussed in detail and the technique given by Gomori (1952). The liberated phosphate ions are precipitated as calcium phosphate which is then demonstrated by a cobalt or silver substitution technique (page 152). The reliability of the Gomori–Takamatsu method has been widely studied and, at the same time, has been the subject of a lively controversy centering on the significance of nuclear staining. While it is now

generally believed that such staining is artifactual and largely due to diffusion and adsorption of enzyme or calcium phosphate, Feigin and Wolf (1957) have presented new evidence favouring the existence of nuclear phosphomonoesterase I. A number of suggestions have been made for minimizing artifacts due to diffusion and adsorption but most of them probably also greatly reduce the enzyme activity (Novikoff, 1955). If a procedure is discovered that does eliminate possible artifacts without decreasing the activity of the enzyme, the Gomori–Takamatsu method will then probably be reliable for demonstrating sites of low enzyme activity. Meanwhile, it appears to be reliable for sites of high activity, requiring only brief incubation times. When incubation must be longer, the results must be interpreted with caution, especially if there is nuclear coloration or if there are sites of high activity near by.

The other method for phosphomonoesterase I is an azo-dye technique, introduced by Menten, Junge and Green (1944) and improved by Manheimer and Seligman (1948). A naphthyl phosphate is used as the substrate. The naphthol liberated by enzyme action is immediately coupled with a diazonium salt to yield an insoluble, intensely coloured pigment. Some of the advantages of the azo-dye technique are that the final product is formed immediately, avoiding the later conversion steps of the first method; the azo-dyes are more insoluble than cobalt sulphide; the formation of pigment can be followed easily and the incubation stopped as soon as desirable; and any preformed deposits of calcium salts or phosphates do not interfere. The important disadvantage of the azo-dye method is that the great instability of the diazonium salts suggested so far necessitates incubating at low temperatures and non-optimum hydrogen-ion concentrations. The results obtained with the azo-dye methods are usually, but not invariably, the same as those obtained with the Gomori–Takamatsu method. It has been suggested that the azo-dye method is relatively insensitive and therefore fails to demonstrate sites of low enzyme activity.

10.22 Phosphomonoesterase II. Phosphomonoesterase II is the designation recommended by Roche (1950) for the enzyme splitting monoesters of orthophosphoric acid in an *acidic* medium. In most tissues, the pH optimum for this enzyme is around

5·0–5·3. Unlike phosphomonoesterase I, this enzyme is unaffected by magnesium ions and is inhibited by fluoride ions. There are two other phosphomonoesterases having acidic pH optima but it is unlikely that they are demonstrated by the histochemical techniques for acid phosphatase.

By correlated biochemical and histochemical studies, Moretti and Mescon (1956) have demonstrated the importance of using different times of incubation in histochemical studies of sites of different phosphomonoesterase II activities.

There are two methods for phosphomonoesterase II, a 'sulphide' and an azo-dye method. In the first, the liberated phosphate is precipitated as lead phosphate which is later converted to lead sulphide (Gomori, 1941). The problems of diffusion and adsorption artifacts are even greater for this method than for the Gomori–Takamatsu method.

The azo-dye method was introduced by Seligman and Manheimer (1949) who used calcium α-naphthyl phosphate as the substrate, coupling the liberated α-naphthol with anthraquinone-1-diazonium chloride. Long periods of incubation are required leading to serious diffusion artifacts and considerable non-specific background staining. The method was improved by Grogg and Pearse (1952) who recommended the sodium salt of the substrate and tetrazotized dianisidine as the coupling agent. Burton (1954) further modified the method so as to retard the rate of hydrolysis by using a non-optimum pH and lowering the substrate concentration, and to accelerate coupling by using a catalyst and increasing the concentration of the coupling agent. In 1955, Rutenberg and Seligman introduced a post-incubation coupling technique using 6-benzoyl-2-naphthyl phosphate as the substrate that is by far the best method to date (Gomori, 1956). The results with this method are quite similar to those with the lead sulphide technique. Minor differences suggest that there might be more than one phosphomonoesterase II and also indicate that there are still significant diffusion artifacts. The results 'must be interpreted with caution, especially with respect to finer details' (Gomori, 1956).

10.23 5-Nucleotidase and phosphamidase. Among the substrate-specific phosphatases, there are two that can be demonstrated histochemically. One of these splits adenosine-5-phosphate when

it is used as the substrate in the Gomori–Takamatsu method at pH 9·2. Gomori (1949*a*) and Newman, Feigin, Wolf and Kabat (1950) have concluded that the enzyme is most probably 5-nucleotidase (Reis, 1934, 1937, 1951). Further studies on this enzyme have been published by McManus, Lupton and Harden (1952), McManus and Lupton (1953), Pearse and Reis (1952), and Wachstein and Meisel (1952, 1954).

The other substrate-specific phosphatase that can be demonstrated histochemically splits *p*-chloroanilido phosphate in Gomori's lead sulphide procedure at pH 5·4–5·8. Gomori (1948, 1949, 1949*a*) concludes that the enzyme is phosphamidase. His method (Gomori, 1952) has been modified by Meyer and Weinmann (1955) to give more consistent and reliable results.

10.3 Aliesterases and cholinesterases

The classification of enzymes hydrolysing various esters has been dealt with in detail by Ammon and Jaarma (1950) and by Augustinnson (1950). The main classes of these enzymes are the aliesterases and the cholinesterases. The aliesterases are subdivided into esterases and lipases. In histochemistry, the term esterases usually refers to substrate-unspecific enzymes that preferentially hydrolyse simple aliphatic esters; while the lipases are considered to be enzymes that hydrolyse glycerol esters of long-chain fatty acids, true fats. The cholinesterases, on the other hand, hydrolyse choline esters more rapidly than other esters and are remarkably readily inhibited by eserine.

In his review of the histochemistry of the esterases, Gomori (1952*a*) suggested a histochemical classification of esterases differing appreciably from the usual biochemical classification of these enzymes. His scheme is based on their behaviour with various substrates.

10.31 Aliesterases. There are three methods for the demonstration of the aliesterases: the 'tween', azo-dye, and indoxyl techniques. The 'tween' method was introduced by Gomori (1945). The 'tweens' are water soluble, long-chained fatty-acid esters of sorbitan or mannitan in which the remaining hydroxyl groups have been etherified with ethylene oxide. Fatty-acid esters of polyethylene glycols can also be used. Upon hydrolysis, the fatty acids are precipitated as their calcium salts. As in Gomori's acid

phosphatase method, the calcium is converted to the lead salt and thence to lead sulphide. Various saturated (stearate, palmitate, and laurate) and unsaturated (oleate, ricinoleate) substrates can be used. The longer-chained esters (for example, stearates) are preferable because, although they are hydrolysed more slowly, they yield a finer crystalline precipitate giving better localization. Using this method, any attempt to differentiate between lipases and esterases should be made cautiously, if at all (Huggins & Moulton, 1948; Nachlas & Seligman, 1949). Gomori (1948) has suggested the use of activators and inhibitors, such as bile-salts, quinine and arsanilate, as aids in differentiation. He has also noted that lipases but not esterases hydrolyse the unsaturated 'tweens' (Gomori, 1949). Around sites of high enzyme activity, with the 'tween' method, there is usually some undesirable diffusion of the enzyme causing cytolysis.

The azo-dye method was introduced by Nachlas and Seligman in 1949, using β-naphthyl acetate as the substrate the liberated β-naphthol being coupled with diazotized α-naphthylamine to form a purplish-red precipitate. Gomori (1950) pointed out that with β-naphthyl acetate there is apt to be considerable diffusion artifact. He recommended using α-naphthyl acetate or naphthol AS acetate under proper conditions of pH and temperature to avoid such artifacts and to obtain sharp cytological localization. With this method taurocholate, for example, can be used to inhibit esterases and permit selective demonstration of lipases (Seligman, Nachlas, Manheimer, Friedman & Wolf, 1949). Using a variety of inhibitors and activators, Gomori and Chessick (1953) demonstrated that there are definite organ and species specificities in the behaviour of the esterases. Gomori (1955) has applied similar methods in his studies on the esterases of human tissues.

Indoxyl acetate was suggested as a substrate by Barrnett and Seligman (1951). It can be hydrolysed by esterases, lipases and cholinesterase yielding the pigment, indigo blue. Essentially all of the cholinesterase is inactivated by fixing in acetone and embedding in paraffin. Taurocholate can be used to inhibit the non-specific esterases so that only lipases are demonstrated. There is great variability in the form of the precipitate. This can be counteracted to some extent by modifying the substrate molecule or adding certain substances to the medium (Holt, 1952).

Localization can be further improved by using a bromo-derivation of the substrate, by controlled promotion of the oxidation using ferricyanide and ferrocyanide, and by using di-indoxyl compounds (Holt & Withers, 1952; Holt, 1956).

10.32 Cholinesterases. The cholinesterases are usually subdivided into the enzymes specifically hydrolysing acetylcholine, variously named 'true' or 'specific' cholinesterases or acetylcholinesterases, and the enzymes non-specifically hydrolysing choline esters, 'pseudo' or 'non-specific' cholinesterases or simply cholinesterases. The common choline esters such as acetylcholine are unsuitable as histochemical substrates because their hydrolysis products are readily soluble and cannot be converted to suitable precipitates. Two types of substrates have been suggested that yield more convenient hydrolysis products: long-chain fatty-acid esters of choline, and esters of thiocholine. The fatty-acid esters are hydrolysed much more slowly than acetylcholine and by only some of the cholinesterases (Glick, 1941). Esters of thiocholine, on the other hand, are hydrolysed by cholinesterases nearly as fast as or faster than the corresponding choline esters.

Following the principle of his 'tween' method for aliesterases, Gomori (1948) developed a method for the cholinesterases using the choline esters of higher fatty acids (C_{12}–C_{16}). The fatty acids liberated by enzymic hydrolysis are precipitated as their cobalt salts because these form smaller crystals than the calcium salts. The cobalt salts are then converted to sulphides. With this method, there are definite organ and species differences in the results obtained with various fatty acid esters of choline and there is no clear-cut differentiation between the 'true' and 'pseudo' cholinesterases (Gomori, 1952).

Koelle and Friedenwalt (1949) introduced the use of acetylthiocholine as a histochemical substrate for the cholinesterases. Koelle (1950; 1951) modified the original procedure to give more satisfactory results. Both acetylcholinesterases and cholinesterases hydrolyse acetylthiocholine. The non-specific enzymes can be inhibited by di-isopropylfluorophosphate so that only acetylcholinesterases are demonstrated with acetylthiocholine. Butryrylthiocholine is hydrolysed by only the non-specific enzymes. With either substrate, the thiocholine formed by hydrolysis is precipitated to copper sulphide. Malmgren and Sylvén (1955) studied

the chemical reactions and the factors influencing them in this method.

An azo-dye method for the cholinesterases has been devised by Ravin, Zacks and Seligman (1953). It gives essentially the same results for the non-specific enzymes as does the revised thiocholine method. Both Barrnett and Seligman (1951) and Holt (1952) have demonstrated the possibility of localizing acetylcholinesterases using indoxylacetate as the substrate.

10.33 Other hydrolytic enzymes. Among the other hydrolytic enzymes, methods have been described for β-D-glucuronidase (Billett & McGee-Russell, 1956; Fishman & Baker, 1957), β-D-galactosidase (Rutenberg, Cohen, Tsou & Seligman, 1952), aryl sulphatase (Rutenberg, Cohen & Seligman, 1952), and aminopeptidase (Gomori, 1954; Burstone & Folk, 1956). The publications cited should be consulted for details and additional references.

10.4 Oxidative enzymes

Baldwin (1952) has discussed the general biochemistry of the oxidative enzyme systems while more detailed accounts are to be found in the appropriate chapters of the monograph edited by Sumner and Myrbäck (1950). The tetrazolium salts have become the most popular reagents for histochemical investigations of enzymes, variously designated as 'dehydrogenases', 'reductases', and 'oxidases' (Verne & Wegmann, 1956). Novikoff (1955) has reviewed some of the problems and limitations in the application of tetrazolium salts to the histochemical study of these enzymes.

Despite the increasing use of tetrazolium salts in the histochemical study of oxidizing enzymes, serious problems of interpretation and localization remain to be settled. For example, exactly what is localized by the reduced tetrazolium? It appears that these reagents can accept hydrogen only from the substrate in the presence of a flavin-containing oxidase or from reduced DPN or TPN in the presence of a flavoprotein enzyme (diaphorase). Thus, it is conceivable that in the case of the specific oxidases, tetrazolium reduction does occur at the site of the oxidative enzyme. The precise localization of a dehydrogenase by tetrazolium reduction requires that the dehydrogenase and its flavoprotein enzyme be located in the same part of the cell and

remain there throughout the test. Cell fractionation studies have shown that this is not the case for every dehydrogenase and its flavoprotein enzyme. Caution should be exercised, therefore, in interpreting tetrazolium reduction as indicating the site of a dehydrogenase. Moreover, differences in results with different substrates cannot be attributed solely to differences in their dehydrogenases. It is possible that at least some of the differences are related to differences in the flavoprotein enzymes or in intermediate steps leading to these enzymes from the dehydrogenases. Farber and his associates (Farber & Bueding, 1956; Farber & Louviere, 1956; Farber, Sternberg & Dunlap, 1956, 1956*a*; Sternberg, Farber & Dunlap, 1956) have studied intensively the use of tetrazolium salts and the interpretation of the results in studies on specific oxidative enzyme activities.

Apart from problems of localization and interpretation related to the exact mechanism of tetrazolium reduction, there are additional problems of localization related to technique. The thick sections that must be used in most instances can conceivably impede penetration of the substrate and reagents and certainly make precise determination of intracellular localization difficult if not impossible. During the preparation of the tissues and the conduct of the test, redistribution of the intact enzyme system or disruption of the morphological organization of its components, and diffusion of the enzyme and the formazan can occur, leading to a decrease or loss of activity or to erroneous localization. Solution of formazan in intra- or extracellular lipid droplets is another source of error.

While a number of tetrazolium salts have been suggested as reagents for oxidizing enzymes, three have been used most generally: triphenyl tetrazolium, neotetrazolium, and blue tetrazolium. Shelton and Schneider (1952) preferred neotetrazolium because it is reduced rapidly and uniformly and its formazan yields the smallest crystals. Atkinson, Melvin and Fox (1950) have reported that iodo-derivatives such as 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium are more resistant to reduction by light and that their formazans tend to diffuse less. A new compound which promises to be a most important addition to this group of reagents has been introduced by Nachlas, Tsou, de Sousa, Chen and Seligman (1957). It is a *p*-nitrophenyl-substituted ditetrazole related to blue tetrazolium, called 'nitro-BT'.

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Addenda

Nile blue test (page 74). The Nile blue solutions, acetic acid, and wash water should be first warmed to the same temperature, 37° or 60° C., before carrying out the test.

1. Bring a section to water. Immerse in 1% aqueous Nile blue for 5 minutes. Wash quickly in water. Immerse for 30 seconds in 1% acetic acid. Wash in water and mount in Farrant's or similar medium.

2. Stain and differentiate a second section exactly as above. Then re-stain it using 0·02% aqueous Nile blue for 5 minutes. Wash quickly and differentiate for 30 seconds in 1% acetic acid. Wash and mount.

0·05 M. Barbitone buffers	**pH 7·4**	**pH 8·5**
2·06% barbitone sodium	50 ml.	50 ml.
0·1N. hydrochloric acid	33 ml.	7·5 ml.
Distilled water	17 ml.	42·5 ml.
see pages	134	133, 142

0·2M. Borate buffers	**pH 8·5**	**pH 9·0**
1·24% boric acid	50 ml.	20 ml.
1·9% borax	50 ml.	80 ml.
see pages	142	140

0·2M. Phosphate buffers	**pH 7·4**	**pH 7·6**
2·76% $NaH_2PO_4.H_2O$	19 ml.	13 ml.
5·36% $Na_2HPO_4.7H_2O$ or		
7·16% $Na_2HPO_4.12H_2O$	81 ml.	87 ml.
see pages	144	135

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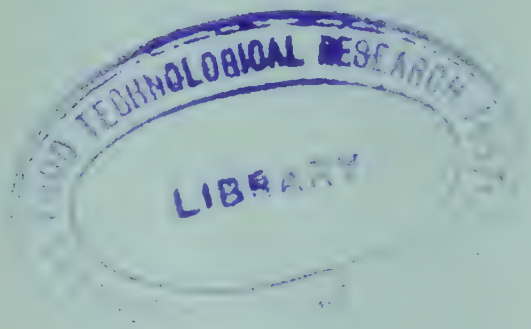

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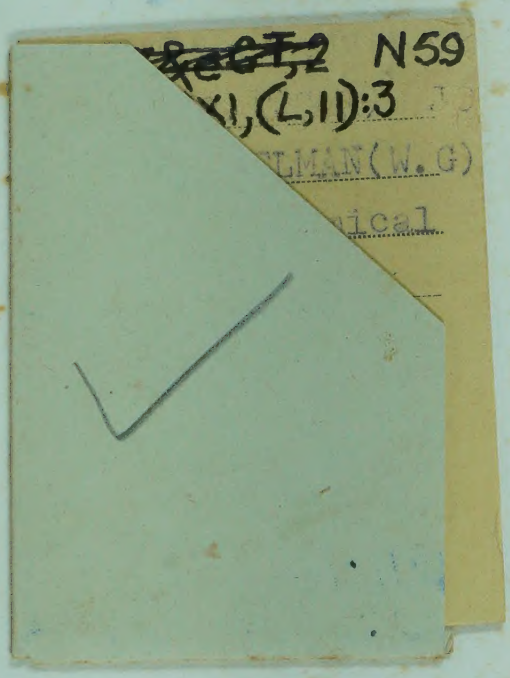

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